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FACULTAD DE VETERINARIA

TESIS DOCTORAL

Influencia de factores nutricionales sobre la expresión génica y la composición tisular en cerdo ibérico

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid

UNIVERSIDAD COMPLUTENSE MADRID

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INFLUENCIA DE FACTORES NUTRICIONALES SOBRE LA EXPRESIÓN GÉNICA Y LA COMPOSICIÓN TISULAR EN CERDO IBERICO

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RITA MARÍA BENÍTEZ YÁÑEZ

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RITA MARÍA BENÍTEZ YÁÑEZ

Realizada bajo la dirección de la doctora
CRISTINA ÓVILO MARTÍN
MADRID, 2019

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El trabajo experimental que ha dado lugar a esta memoria ha sido realizado en el Departamento de Mejora Genérica Animal del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, financiado mediante los siguientes proyectos de investigación:

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"La confianza en sí mismo es el secreto del éxito"
Ralph Waldo Emerson

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ABREVIATURAS UTILIZADAS

ACACA	Acetyl-CoA carboxylase alpha (Acetil-CoA carboxilasa, alfa)
ACOX1	Acyl-CoA oxidase 1, palmitoyl (Acil-CoA oxidasa 1)
ACSL4	Acyl-CoA synthetase long-chain family member cuatro (Acil-CoA sintasa de ácidos grasos de cadena larga 4)
ADN	Ácido desoxirribonucleico
AdPLA	Adipose-specific phospholipase A2 (Fosfolipasa A2 especifica de tejido adiposo)
AEAT	Agencia Española de Administración Tributaria
AG	Ácidos grasos
AGMI	Ácidos grasos Monoinsaturados
AGPI	Ácidos grasos Poliinsaturados
AGRP	Agouti-related protein (Proteína asociada a agutí)
AGS	Ácidos grasos Saturados
ARN	Ácido ribonucleico
ASXL2	Additional sex combs-like protein
ATGL	Adipose triglyceride lipase (lipasa de triglicéridos del tejido adiposo)
ATP	Adenosin trifosfato
BACs	Cromosomas artificiales de bacterias
BF	*Biceps femoris*
CART	Cocaine and amphetamine regulated transcript (Transcrito regulado por anfetamina y cocaína)
CEBPα, β, δ	CCAAT/enhancer binding protein, alpha, beta, delta (Proteínas de unión a CCAAT/enhancer)
CH	Carbohydrates diet (dieta con carbohidratos como fuente de energía)
CHOP	CCAAT-enhancer-binding protein homologous protein (Proteína homóloga a las proteínas de unión a CCAAT)
CPT-1	Carnitine-palmitoyl-transferase 1 (Carnitina pamitoil transferasa 1)
DE	Diferencialmente expresado
DEGs	Genes diferencialmente expresados
DGAT1	Diacylglycerol O-acyltransferase 1 (Diacilglicerol aciltransferasa 1)
DLK1	Delta-like 1 homolog (drosophila) (Homólogo de la proteína similar a delta)

DU	Duroc
EGR2	Early growth response protein 2 (proteína de respuesta de crecimiento temprano 2)
ELOVL6	Long-chain fatty-acyl elongase (Elongasa de ácidos grasos de cadena
FABP4	Fatty acid binding protein 4 (Proteína 4 de unión a ácidos grasos)
FABP5	Fatty acid binding protein 5 (Proteína 5 de unión a ácidos grasos)
FAD	Flavín adenín dinucleótido o dinucleótido de flavina y adenina
FASN	Fatty acid synthase (Sintasa de ácidos grasos)
FC	Fold-Change (valor de cambio en la expresión)
G3PDH	Glycerol-3-phosphate dehydrogenase (Glicerol-3-fosfato deshidrogenasa)
G6PC	Glucose-6-phosphatase, catalytic subunit (glucosa 6-fosfatasa alfa)
GIM	Grasa intramuscular
GLUT4	Glucose transporter (Transportador de glucosa)
GWAS	Genome wide association study (Estudio de asociación del genoma)
HO	High Oleic diet (dieta enriquecida en girasol alto oleico)
HSL	Hormone-sensitive lipase (lipasa sensible a las hormonas)
IB	Ibérico
IFNG	Interferon, gamma
IGF1	Insulin-like growth factor 1 (Factor de crecimiento similar a la insulina 1)
IGF2	Insulin-like growth factor 2 (Factor de crecimiento similar a la insulina 2)
INE	Instituto Nacional de Estadística
INS	Insulin (Insulina)
INSIG1	Insulin induced gene 1(gen 1 inducido por insulina)
INSR	Insulin receptor (Receptor de la insulina)
KLF5	Kruppel-like factor 5 (Factor similar a kruppel)
LD	*Longissimus dorsi*
LEP	Leptin (Leptina)
LEPR	Leptin receptor (Receptor de leptina)
LPL	Lipoprotein lipase (lipoproteína lipasa)
LW	Large White
LXRs	Liver x receptor (Receptor hepático x)

MAPA	Ministerio de Agricultura Pesca y Alimentación
MAPAMA	Ministerio de Agricultura, Pesca, Alimentación y Medio Ambiente
MC4R	Melanocortin cuatro receptor (Receptor de melanocortina 4)
ME1	Malic enzyme (Enzima málico)
MGLL	Monoacylglycerol lipase (lipasa de monoacilglicerol)
mRNA	ARN mensajero
MS	Meishan
NAD^+	Nicotinamida adenina dinucleótido fosfato forma oxidada
NADPH	Nicotinamida adenina dinucleótido fosfato forma reducida
NPY	Neuropetpide Y (Neuropéptido Y)
PCK1	Phosphoenolpyruvate carboxykinase 1 (carboxicinasa de fosfoenopiruvato 1 (soluble))
PCK2	Phosphoenolpyruvate carboxykinase 2 (carboxicinasa de fosfoenolpiruvato 2)
PCR	Polymerase chain reaction (Reacción en cadena de la polimerasa)
PDGF	Platelet-derived growth factor beta polypeptide (Factor de crecimiento derivado de plaquetas)
PKA	Protein kinase cAMP-dependent (proteina quinasa A)
POMC	Propiomelanocortin
PPARG	Peroxisome proliferator-activated receptor gamma (Receptores activados por proliferadores peroxisomales gama)
PTGS	Prostaglandin-Endoperoxide Synthase 1 (prostaglandina-endoperóxido sintasa 1)
qPCR	Quantitave PCR (Polymerase chain reaction) (PCR (Reacción en cadena de la polimerasa) cuantitativa)
QTLs	Quantitative trait locus (Locus de caracteres cuantitativos)
RARA	Retinoic acid receptor alpha (Receptor de ácido retinoico)
RIBER	Registro informático del Ibérico
RNA-Seq	RNA sequencing (Secuenciación del ARN)
RXRA	Retinoid x receptor alpha (Receptor retinoide x)
RXRG	Retinoid x receptor gamma (Receptor retinoide x)
SCD	Stearoyl-CoA desaturase/delta-9-desaturase (Estearoil CoA desaturasa)
SGSC	Swine Genome Sequencing Consortium (Consorcio para la secuenciación del genoma del cerdo)

SNP	Single nucleotide polymorphism (Polimorfismo de un solo nucleótido)
SREBF1	Sterol regulatory element-binding transcription factor 1(proteína de unión al elemento regulador de esterol 1)
SREBP1C	Sterol regulatory element binding protein 1c (Proteína de unión al elemento regulador del esterol 1c)
TA	Tejido adiposo
TG	Triglicéridos
TGFB	Transforming growth factor beta (factor de crecimiento transformante)
TNFα	Tumor necrosis factor (factor de necrosis tumoral)
UCP3	Mitochondrial uncoupling protein 3 (proteína de desacoplamiento mitocondrial 3)
UE	Unión Europea
USDA	U.S. Department of Agriculture

RESUMEN/ABSTRACT

RESUMEN

El cerdo ibérico se caracteriza por un crecimiento poco eficiente, un escaso desarrollo muscular y una gran capacidad de acumulación de grasa, además de una gran adaptación al medio y rusticidad. Al igual que otras razas autóctonas tradicionales, su rendimiento es menor al de las razas seleccionadas, lo que suele ir asociado a una menor rentabilidad. Su producción es un claro ejemplo de sistema productivo sostenible orientado a la obtención de productos cárnicos de alta calidad, en el que, además de los aspectos genéticos, los aspectos ambientales y de manejo (especialmente los nutricionales) tienen una influencia decisiva en la composición tisular y en la calidad de los productos. Debido a su bajo rendimiento productivo y con el objetivo de mejorarlo se ha cruzado de forma habitual con la raza duroc, siendo la mayoría de la producción del sector del ibérico correspondiente a cebo intensivo y cruce con duroc. Aunque numéricamente su producción sea menor, la producción de animales 100% ibéricos y de bellota es más relevante, por su excelente calidad y su gran valor económico.

Su sistema tradicional de producción está basado en la restricción de alimento previo al engorde y en la ingesta de bellotas y pasto en el periodo final del cebo (montanera), lo que conduce a un cambio en el perfil lipídico del músculo y de la grasa caracterizado por un aumento en el contenido de ácido oleico. Este perfil proporciona unas características organolépticas y tecnológicas especiales a su carne e influye notablemente en la calidad excelente de sus productos curados. En la producción porcina ibérica en regímenes de campo o intensivos, las dietas suplementadas con AG, en especial con ácido oleico, se han utilizado como estrategia para conseguir una monoinsaturación de los tejidos que mimetice el sistema tradicional de engorde en montanera. El empleo de dietas enriquecidas en distintos tipos de AG o distintas fuentes de energía se ha estudiado en esta tesis desde un enfoque nutrigenómico, investigando los efectos que los AG de la dieta tienen sobre el fenotipo y que están vehiculados por cambios en la expresión génica.

Los objetivos principales de la presente Tesis Doctoral han sido profundizar en el conocimiento de los mecanismos moleculares y los genes implicados en la regulación de los procesos de lipogénesis, lipolisis y el metabolismo lipídico, así como comprender el efecto de la incorporación de distintas fuentes de energía sobre la composición tisular y la expresión génica en el cerdo ibérico. Además,

los trabajos han estado enfocados a la comprensión de los efectos conjuntos de la dieta y de la raza (ibérico y duroc) sobre el metabolismo tisular y el fenotipo, para conocer la influencia de las interacciones genética-nutrición sobre distintos procesos fisiológicos y metabólicos con trascendencia a nivel productivo.

Para la consecución de estos objetivos se diseñaron 3 experimentos. En el primer diseño experimental se estudiaron los efectos de la composición de ácidos grasos (AGS y AGPI) de la dieta de cerdos ibéricos en fase de cebo, sobre el perfil de AG y sobre la expresión de genes codificantes para enzimas clave relacionadas con la adipogenesis, lipogenesis y el metabolismo lipídico, en los tejidos adiposo, muscular y hepático. En el segundo diseño experimental se evaluó el efecto de la fuente de energía de la dieta, comparando una dieta con carbohidratos (CH) frente a una dieta enriquecida con un 6% de aceite de girasol alto oleico (HO), sobre la transcripción de un panel de genes candidato en tejido adiposo del jamón, en diferentes períodos de crecimiento y en situación de ayuno moderado (18h). El tercer experimento se diseñó para comprender los efectos conjuntos de la dieta, la raza y el ayuno, así como su interacción, con el fin de mejorar el conocimiento de los genes y rutas metabólicas implicadas en las diferencias entre razas y en el efecto de la dieta, así como los aspectos diferenciales de la respuesta a la dieta entre razas. Para ello se emplearon las razas ibérica y duroc en pureza, mantenidas en idénticas condiciones de manejo durante la fase de crecimiento, y se combinaron las estrategias de gen candidato y de estudio del transcriptoma. Se estudiaron los efectos de la raza (Ibérica *vs* Duroc), un período de ayuno largo (24 h), y la fuente de energía de la dieta (HO *vs* CH), así como sus interacciones, sobre la composición del tejido adiposo y la expresión génica.

Los resultados indicaron que, a corto/medio y largo plazo, las intervenciones nutricionales con distinta fuente de energía inducen adaptaciones transcripcionales en los tejidos adiposo y hepático. Los resultados fueron coherentes en los distintos tiempos y experimentos analizados y fueron compatibles con una activación de la síntesis endógena *de novo* de AG en el grupo suplementado con carbohidratos y activación de la desaturación de AG en los animales suplementados con grasa saturada. Los AGMI y AGPI de la dieta mostraron una importante deposición directa en los tejidos. La expresión de genes codificantes de enzimas lipogénicas aumentó con la edad, mientras que el regulador *PPARG* mostró mayor expresión en tejidos de animales jóvenes, en concordancia con su función regu-

ladora de procesos tempranos de diferenciación de adipocitos. Por otra parte, se observó que la regulación de los genes lipolíticos en el tejido adiposo, por factores como la raza, el estado de alimentación y la dieta, es muy compleja debido a que está sujeta a intrincadas interacciones.

Los estudios realizados también nos han proporcionado una caracterización fenotípica y transcripcional de los procesos del metabolismo lipídico en el tejido adiposo de cerdos ibéricos y duroc puros en crecimiento, criados en idénticas condiciones experimentales, que coincide con el alto potencial lipogénico y de desaturación, el elevado apetito, el genotipo ahorrador y la resistencia a la leptina de la raza ibérica. Además, los resultados de expresión génica relativos al efecto del ayuno indicaron una persistencia de la lipogénesis *de novo* durante el ayuno en cerdo ibérico, en consonancia con su fenotipo graso.

El estudio global del transcriptoma del tejido adiposo mostró un profundo efecto de la raza, así como un menor y más complejo efecto de la dieta que está fuertemente modulado por la raza. La mayoría de las diferencias transcripcionales que encontramos entre razas se relacionaron con el crecimiento, la formación de matriz extracelular, el metabolismo de los lípidos y de los carbohidratos y de forma remarcada con la respuesta inflamatoria e inmune. Los resultados sugirieron el desarrollo de una inflamación crónica de bajo grado y un síndrome de resistencia a la insulina en el tejido adiposo de los animales ibéricos a pesar de su corta edad, así como un metabolismo lipídico más intenso, todo ello concordante con su muy superior deposición grasa. En cambio, los genes sobreexpresados en la raza duroc indicaron la activación de rutas involucradas en el crecimiento del animal y el desarrollo de la estructura tisular. Los efectos de la dieta y su interacción con la raza indicaron una respuesta más intensa a la dieta en los cerdos ibéricos, relacionada esencialmente con cambios en los genes implicados en la respuesta inflamatoria e inmune, el metabolismo de los lípidos y el engorde. Los resultados permitieron también poner de manifiesto la importancia de estos efectos de interacción así como la dificultad de su estudio a nivel de transcriptoma.

La caracterización fenotípica de los grupos experimentales aporta una información novedosa e importante sobre la utilidad de las intervenciones nutricionales empleadas para mejorar la calidad de los productos del cerdo, no solo en la raza ibérica sino también en la raza duroc. La exploración de los efectos transcripcionales de la raza, las intervenciones con diferentes fuente de energía, el período

de crecimiento y la situación fisiológica del animal, así como sus interacciones, han contribuido a profundizar en la comprensión de la regulación del metabolismo y la lipogénesis en la raza ibérica y apoyan su utilidad como modelo animal para estudiar la obesidad y trastornos metabólicos, incluyendo el estudio de los procesos inflamatorios asociados a la acumulación de grasa en el tejido adiposo.

ABSTRACT

The Iberian pig is characterized by low growth efficiency, poor muscle development and a high capacity for fat accumulation, as well as a good adaptation to the environment and rusticity. As other traditional autochthonous breeds, its productive yield is lower than in selected breeds, which is usually associated with low profitability. The Iberian pig production is a clear example of a sustainable production system aimed at obtaining high quality meat products, in which, in addition to genetic aspects, environmental and management aspects (especially nutritional ones) have a decisive influence on tissue composition and products' quality. Due to its low productive yield and with the aim of improving it, the Iberian pig has been usually crossed with the Duroc breed, with the majority of the production of the Iberian sector corresponding to intensive fattening and Duroc crossbreeding. Although numerically lower, the production of purebred Iberian and acorn-fed animals is more relevant, due to its excellent quality and great economic value.

Its traditional production system is based on the restriction of feed before fattening and on the intake of acorns and grass at the final fattening period ("*montanera*"), which leads to a change in the lipid profile of muscle and fat, characterized by an increase in oleic acid content. This profile provides special organoleptic and technological characteristics to its meat and notably influences the excellent quality of its dry-cured products. In Iberian pig production in extensive ("*cebo de campo*") or intensive systems, the use of diets supplemented with FA, especially with oleic acid, has been used as an approach to achieve a monounsaturation in the tissues that mimics the traditional system of fattening in "*montanera*". The use of diets enriched in different FA or different energy sources has been studied in this thesis from a nutrigenomic perspective, investigating the effects that dietary FA have on the phenotype and which are due to changes in gene expression.

The main goals of this Doctoral Thesis have been to deepen in the knowledge of the molecular mechanisms and genes involved in the regulation of the processes of lipogenesis, lipolysis and lipid metabolism, as well as to understand the effect of the incorporation of different energy sources on tissue composition and gene expression in the Iberian pig. In addition, the works have been focused on understanding the joint effects of diet and breed (Iberian and Duroc) on tissue

metabolism and phenotype, in order to know the influence of genetics-nutrition interactions on different physiological and metabolic processes with significance at the productive level.

In order to achieve these objectives, three experiments were designed. In the first experimental design, the effects of the dietary FA composition (SFA and PUFA) on the FA profile and on the expression of key genes involved in adipogenesis, lipogenesis and lipid metabolism were studied, in adipose, muscular and hepatic tissues of fattening Iberian pigs. In the second experimental design, the effect of diet energy source was evaluated, comparing a diet with carbohydrates (CH) against a diet enriched with 6% high oleic sunflower oil (HO), on the transcription of a panel of candidate genes in ham adipose tissue, in different periods of growth and after a moderate fasting (18h). The third experiment was designed to understand the joint effects of diet, breed and fasting, as well as their interaction, in order to improve the knowledge of the genes and metabolic pathways involved in breed and diet effects, as well as the differential aspects of the response to diet between breeds. Pure Iberian and Duroc pigs, maintained in identical management conditions during the growth phase, were used, and a combination of the candidate gene and the transcriptome approaches was applied. Breed effects (Iberian vs Duroc), a long fasting period (24 h), and diet energy source (HO vs CH), as well as their interactions, were studied on adipose tissue composition and gene expression.

The results indicated that, in the short/medium and long terms, nutritional interventions with different energy sources induce transcriptional adaptations in adipose and hepatic tissues. The results were consistent in the different times and experiments analyzed, and agreed with an activation of the endogenous *de novo* synthesis of FA in the carbohydrate-supplemented group and activation of the FA desaturation in animals supplemented with saturated fat. The dietary MUFA and PUFA showed an important direct deposition in the tissues. Expression of lipogenic genes increased with age, while the *PPARG* regulator showed greater expression in growing animals, in accordance with its regulatory function on early adipocyte differentiation processes. On the other hand, the regulation of lipolytic genes in adipose tissue by factors such as breed, feeding status and diet was shown to be very complex and subjected to intricate interactions.

The studies have also provided a phenotypic and transcriptional characterization of lipid metabolism processes in adipose tissue of growing Iberian pigs and Duroc

pure pigs, bred under identical experimental conditions, which agrees with the high lipogenic and desaturation potential, high appetite, thrifty genotype and leptin resistance of the Iberian breed. In addition, gene expression results related to the fasting effect indicated a persistence of *de novo* lipogenesis during fasting in Iberian pigs, in agreement with their fat phenotype.

The global study of adipose tissue transcriptome shows a profound effect of breed, as well as a smaller and more complex diet effect which is strongly modulated by breed. Most transcriptional differences found between breeds were related to growth, extracellular matrix formation, lipid and carbohydrate metabolism and remarkably to inflammatory and immune response. Results suggested the development of chronic low-grade inflammation and an insulin resistance syndrome in the adipose tissue of Iberian animals, despite their young age, as well as a more intense lipid metabolism, consistent with their much higher fat deposition. In contrast, the overexpressed genes observed in Duroc breed indicated the activation of pathways involved in animal growth and tissue structure development. Diet effect and its interaction with breed indicated a more intense response to diet in Iberian pigs, essentially related to changes in genes involved in the inflammatory and immune response, lipid metabolism and fattening. The results also made it possible to highlight the relevance of these interaction effects as well as the difficulty of their study at transcriptome level.

The phenotypic characterization of the experimental groups provides novel and important information on the usefulness of nutritional interventions to improve pork products quality, not only in Iberian breed but also in Duroc. The exploration of the transcriptional effects conditional on breed, nutritional interventions with different energy sources, growth period and animal feeding status, as well as their interactions, have contributed to deepen in the understanding of metabolism and lipogenesis regulation in the Iberian breed. These results support its usefulness as animal model to study obesity and metabolic disorders, including the study of inflammatory processes associated with fat accumulation in adipose tissue.

1. INTRODUCCIÓN

1.1. ORIGEN DEL CERDO DOMÉSTICO Y SUS RAZAS

El cerdo es un mamífero euteriense miembro de la familia *Suidae,* del orden *Cetartiodactyla*, compuesto por un grupo de especies que se originaron hace entre 20 y 30 millones de años (Ruvinsky *et al*., 2011; Frantz *et al*., 2016). De esta familia, *Sus scrofa* (jabalíes y cerdos domésticos) es la única especie domesticada.

Los cerdos salvajes se originaron en el sudeste de Asia hace unos 3 o 4 millones de años y posteriormente se extendieron por casi todo el continente asiático y Europa. Diversos estudios revelan una profunda división filogenética entre los jabalíes europeos y asiáticos. Esta divergencia se ha estimado en cerca de un millón de años a partir de secuencias genómicas y del análisis del genoma mitocondrial (Groenen *et al*., 2016; Fernández *et al*., 2011).

En cuanto al cerdo doméstico (*Sus scrofa domestica*), evidencias arqueológicas y genéticas sugieren que tuvo su origen a partir de jabalíes. La estimación del reloj molecular indica que la división entre los dos grupos asiático y europeo es significativamente anterior a la evidencia de domesticación, lo que sugiere independencia en los eventos de domesticación en cada área desde linajes divergentes de jabalí (Larson *et al*., 2005, Groenen *et al*., 2016).

De todos los suidos, solo los jabalíes han logrado extenderse en varios continentes, lo que demuestra que son extremadamente adaptables a una amplia gama de entornos y climas. En ese sentido, es interesante destacar que el jabalí es el único suido domesticado. Esto puede deberse simplemente a su amplia distribución a través de Eurasia, teniendo contacto directo y frecuente con humanos, pero también es posible que la adaptabilidad extrema de esta especie haya contribuido a su domesticación. La domesticación del cerdo tuvo lugar hace unos 9000 a 10,000 años de forma independiente en dos lugares: en el este de Anatolia y en China. Siguiendo estas domesticaciones iniciales, los cerdos pronto acompañaron a los agricultores a medida que se extendieron desde el este de Anatolia a Europa y en toda China. Esto se ha podido deducir del análisis de los genomas mitocondriales porcinos (Larson *et al.*, 2005; Larson *et al.*, 2010) y más recientemente de los datos de secuenciación del genoma completo (Frantz *et al*., 2015), determinando que la domesticación ha sido un proceso gradual y continuado en el tiempo y que implica una mezcla repetida y un flujo continuo de genes de los jabalíes a las poblaciones domesticadas. Por lo tanto, la domes-

ticación de los cerdos no debería ser considerada como una serie de eventos fijos que ocurrieron hace 10.000 años, sino como un proceso gradual. Los jabalíes se sintieron atraídos por los asentamientos humanos como una forma fácil de conseguir alimentos, y fue después de milenios cuando los humanos empezaron a tener cerdos como una especie verdaderamente domesticada. Este proceso ocurrió en Europa y China a ritmos muy diferentes, con cerdos mantenidos en recintos dentro de los asentamientos humanos en una etapa relativamente inicial en China, mientras que en Europa hasta el final de la Edad Media, gran parte de los cerdos domésticos pastoreaban libremente por los bosques como rebaños asilvestrados (White, 2011).

Durante los siglos XVII y XVIII, en respuesta al aumento creciente de la demanda de productos cárnicos se produjo un cambio importante en la producción porcina (White, 2011; Porter, 1993). Fue entonces cuando la agricultura del norte de Europa se expandió a partir de la puesta en cultivo de amplias áreas forestales. Las razas asiáticas fueron importadas para crear variedades mejoradas primero en Inglaterra y después en América (Giuffra *et al*., 2000). Estas nuevas razas y cruces combinaban el gran tamaño y crecimiento tardío de los cerdos del norte de Europa con la mayor precocidad inicial (crecimiento temprano) y mayor contenido graso de las razas asiáticas (White, 2011), y desempeñaron un papel vital en la transformación del cerdo desde un animal de subsistencia a un productor industrial de carne y grasa. A partir de entonces, las diversas razas porcinas fueron seleccionadas de acuerdo con criterios específicos de diferentes grupos de criadores, originando un gran número de razas porcinas especializadas, con fenotipos muy diferentes. Procesos similares realizados en otros países dieron lugar a un gran número de razas diferentes. Desde la domesticación hasta las prácticas de crianza actuales, las prácticas productivas y de selección han influido profundamente en los genomas de los cerdos domésticos (Groenen *et al*., 2012).

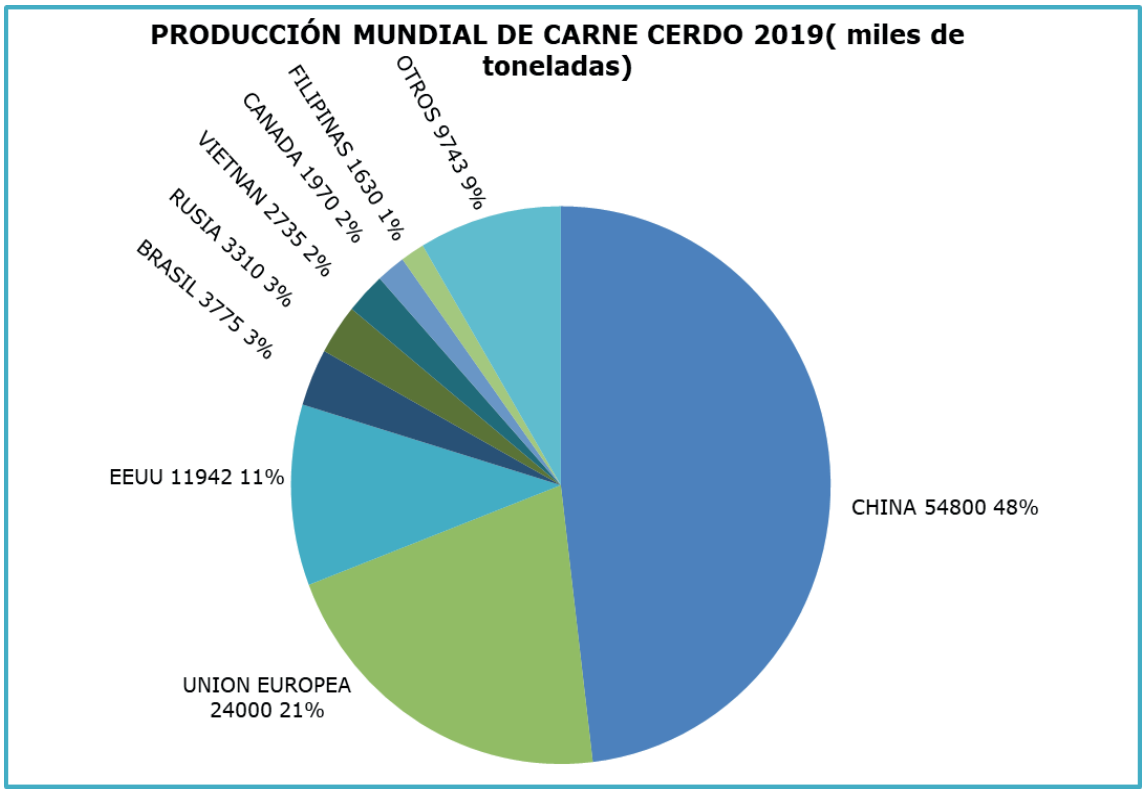
En la actualidad, pese a la notable disminución experimentada en las últimas décadas, existen 350 razas de cerdo recogidas en la página web del Instituto Internacional de Investigación Ganadera (http://agtr.ilri.cgiar.org/index.php?option=com_content&view=article&id=240&Itemid=298). La mayoría de estas razas actuales son razas locales, mientras que solo unas pocas razas están intensamente seleccionadas y se utilizan en la industria porcina a nivel mundial, siendo las más extendidas (Ollivier *et al*., 2009).

1.2. LA INDUSTRIA PORCINA EN LA ACTUALIDAD

El sector porcino español tiene una importancia clave en la economía de nuestro país ya que supone el 12.7 % de la Producción Final Agraria.

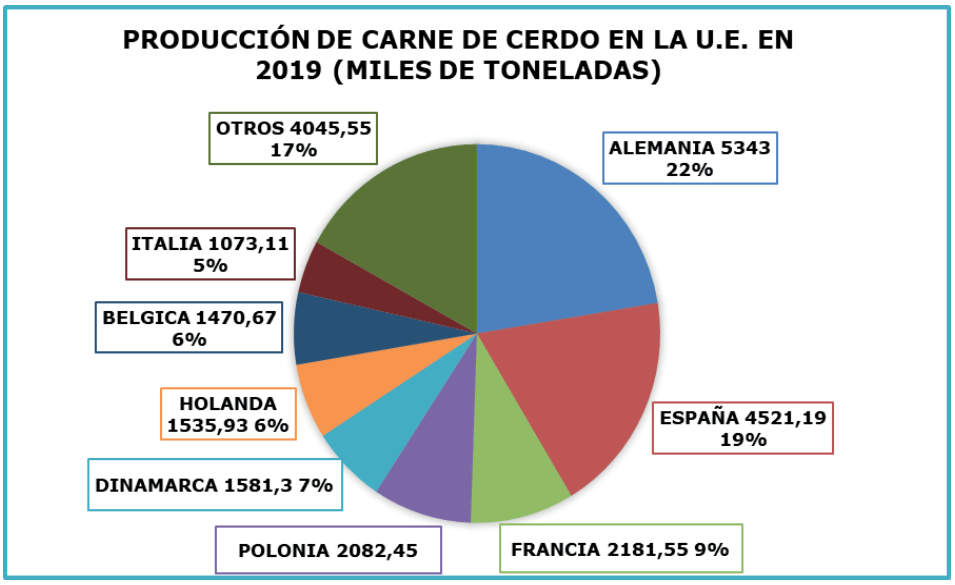
Dentro de las producciones ganaderas, que representan en torno al 38% de la Producción Final Agraria, el sector porcino ocupa el primer lugar en cuanto a su importancia económica alcanzando el 36.4 % de la Producción Final Ganadera (Indicadores económicos del sector porcino 2017, www.mapa.gob.es). A nivel mundial, la UE es el segundo productor de carne de porcino después de China (Figura 1).

Figura 1. Producción mundial de carne de cerdo. Fuentes: USDA Oficina de análisis global del Servicio de Agricultura.



Por países, España es la cuarta potencia productora a nivel mundial (después de China, EEUU y Alemania), mientras que, a nivel europeo, España ocupa el segundo lugar en producción con un 19 % de las toneladas producidas (Fuente: Eurostat 2019), por detrás de Alemania, y es el primer país de la UE en censo, con más del 19 % del censo comunitario (datos 2018, Fuente: Eurostat 2019) (Figura 2).

Figura 2. Producción de carne de cerdo en la U.E. Fuente: Eurostat 2019 Elaboración: Comisión europea de Agricultura y departamento de desarrollo rural.



Durante los últimos años el sector porcino ha crecido notablemente, tanto en producción como en número de animales, gracias al empuje de los mercados exteriores y apoyado a su vez en la competitividad del sector a nivel mundial. Este aumento de la producción, ha incrementado la ya elevada tasa de autoabastecimiento, (174 % en 2017, Fuente: SG Estadística, AEAT, INE) lo que convierte a la exportación en un elemento esencial para el equilibrio del mercado. Con una balanza comercial muy positiva, España se ha consolidado como segundo mayor exportador de porcino de la UE, sólo por detrás de Alemania, aumentando espectacularmente las exportaciones a terceros países, especialmente a China, que se ha convertido en el primer destino de las exportaciones de carne de porcino español en el último periodo. En el contexto internacional, la UE es la principal potencia exportadora (Fuente MAPAMA, www.map.gob.es).

La importancia de la producción española en el conjunto de la UE se ha ido incrementando durante las últimas campañas, puesto que hace solo cinco años la producción española apenas superaba el 15 % del total de la UE. El incremento ha sido consecuencia del espectacular desarrollo del sector en España, claramente por encima de la media de la UE. Así, si durante los últimos cuatro años la producción de carne en la UE ha crecido un 5.2 %, la producción en España ha crecido un 19.9 % en el mismo periodo, lo que da una idea del enorme crecimiento que está experimentando el sector a nivel nacional.

Todo ello pone de manifiesto la gran importancia del sector porcino español a nivel económico y el interés que suscita la investigación, desarrollo e innovación en el marco de la mejora de los principales aspectos productivos de esta especie.

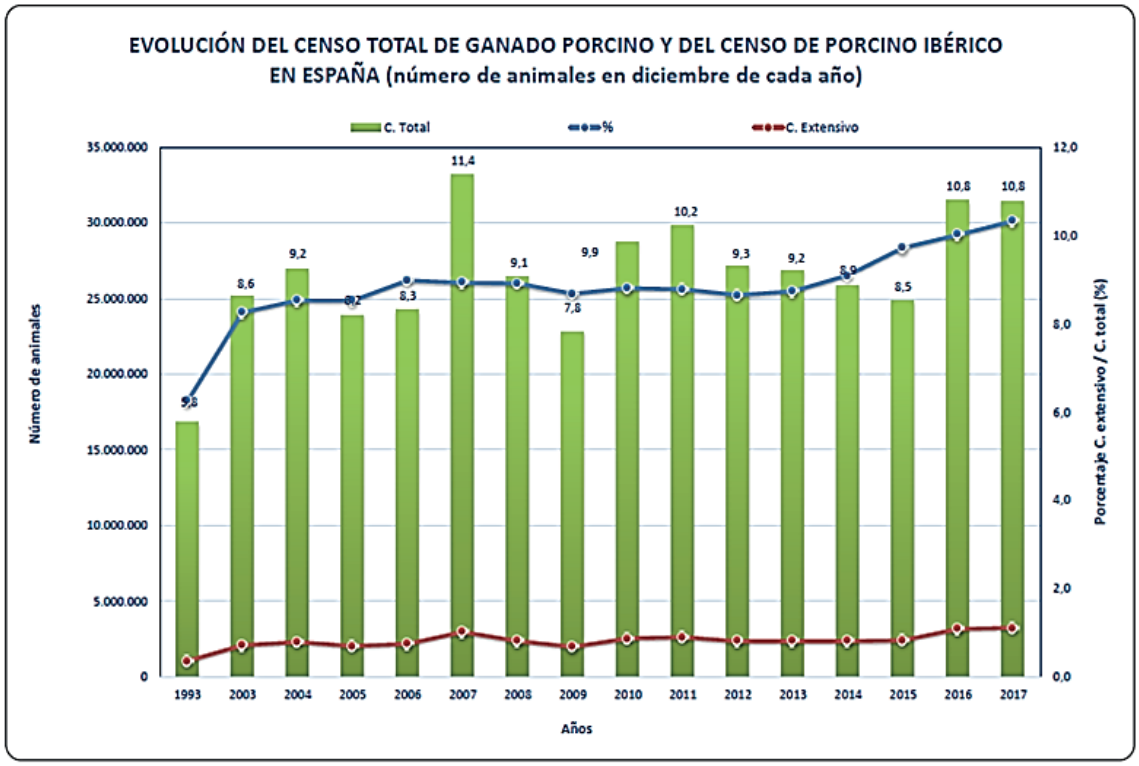
1.2.1. EL CERDO IBÉRICO EN EL SECTOR PORCINO

Dentro del sector porcino español merece una mención especial el cerdo ibérico debido a su tipo de producción, con productos de alto valor económico y un mercado en auge.

A nivel nacional, el censo de porcino ibérico supone aproximadamente el 10.67 % del censo total de porcino a noviembre de 2018 (Fuente: Estadísticas Agrarias Ganadería. Encuestas ganaderas de Porcino 2018. MAPAMA), habiendo aumentado un 3 % sobre el censo nacional en los últimos cuatro años. Extremadura, Andalucía y Castilla y León albergan el 95.3 % del censo nacional de porcino ibérico (más de 3 millones de animales), con una distribución respecto al total del 37.5 %, 29.5 % y 28.3 %, respectivamente. A nivel provincial, cabe destacar que Badajoz representa un 33 % y Salamanca un 16.4 % del censo ibérico nacional total.

Desde 2014 el censo ha experimentado una recuperación importante a pesar del descenso gradual que había tenido en años anteriores, influenciado por la recuperación económica que ha hecho que aumente notablemente la demanda de sus productos de alta calidad (Figura 3).

Figura 3: Evolución del censo porcino y porcino ibérico a diciembre de 2017. Fuente: S.G. Estadística. Elaboración: S.G. Productos Ganaderos



1.3. LAS RAZAS IBERICA Y DUROC

El cerdo ibérico deriva del *Sus scrofa mediterraneous* (Aparicio Sánchez, 1960; Dieguez, 1992), y se caracteriza por presentar una elevada rusticidad, alto potencial lipogénico, gran capacidad de desaturación, alto nivel de veteado en sus músculos y una gran resiliencia (López-Bote, 1998). Además posee una gran variabilidad, debido a la tardía definición de esta raza, ausencia de prototipo racial y al escaso flujo genético hasta las recientes variedades (Fabuel *et al*., 2004). La raza ibérica está bien diferenciada genéticamente respecto a otras razas autóctonas y europeas y no presenta introgresión de genes asiáticos en su acervo genético (Alves *et al*., 2003; Muñoz *et al*., 2018).

Se considera una "agrupación racial" autóctona del sudoeste de la Península Ibérica tradicionalmente estructurada en estirpes y líneas, con presencia de variedades dominantes negras, entrepeladas y retintas con notable diferenciación genética y productiva (Fabuel *et al*., 2004; Benito *et al*., 2000). Solo recientemente, se han iniciado programas de selección de nuevas líneas de cerdo ibérico (Ibáñez-Escriche *et al*., 2016). La línea Torbiscal (Rodríguez *et al.,* 2019), que es con la que se ha trabajado en esta tesis, se obtuvo en 1963 mediante la fusión de cuatro estirpes de cerdo ibérico preservadas por criadores privados, y que desde 1944-45 se mantuvieron con registros genealógicos y productivos en el rebaño experimental de "El Dehesón del Encinar" (Oropesa, Toledo, España). Se escogieron cuatro antiguas ganaderías, que llevaban mucho tiempo de cría aislada, y representaban la gran dispersión de tipos de cerdos ibéricos entonces existentes. En cada una de ellas se eligió y adquirió un lote, llegando los animales a Oropesa en 1944 y 1945, quedando la piara cerrada a partir de este contingente fundacional: Dos de estas estirpes eran coloradas y provenían de las piaras portuguesas del Conde de Ervideira (Evora) y el Sr. Picao Caldeira (Elvas). Las otras negras sin pelo, provenían de las piaras de los hermanos Donoso (Campanario) y el Sr. Fabián Lozano (Puebla de la Calzada) (Odriozola, 1976) (Figura 4). La última estirpe (llamada hoy Guadyerbas) y la línea Torbiscal se conservan actualmente en un programa de conservación del INIA. Las cuatro cepas fundadoras citadas fueron elegidas como representativas de las principales variedades existentes en ese momento: la variedad dorada del Alentejo (Ervideira), la variedad castaña (Caldeira) y las dos variedades sin pelo: el Campanario óseo y el Puebla primitivo.

Figura 4. (Silió *et al.,* 2013) "Una experiencia irrepetible: la piara de cerdos ibéricos de 'El Dehesón del Encinar' (1944-2012)". XV Jornadas sobre Producción Animal. Zaragoza

Ervideira
Dourado alentejano
6 M y 17 H

Caldeira
Retinto
5 M y 14 H

Campanario
Negro lampiño de La Serena
4 M y 24 H

Puebla
Negro lampiño del Guadiana
5 M y 27 H

En los últimos años se ha producido un importante proceso de cruces entre estirpes ibéricas, que hace que actualmente la mayor parte de los reproductores tengan un origen mixto (Alves *et al*., 2006).

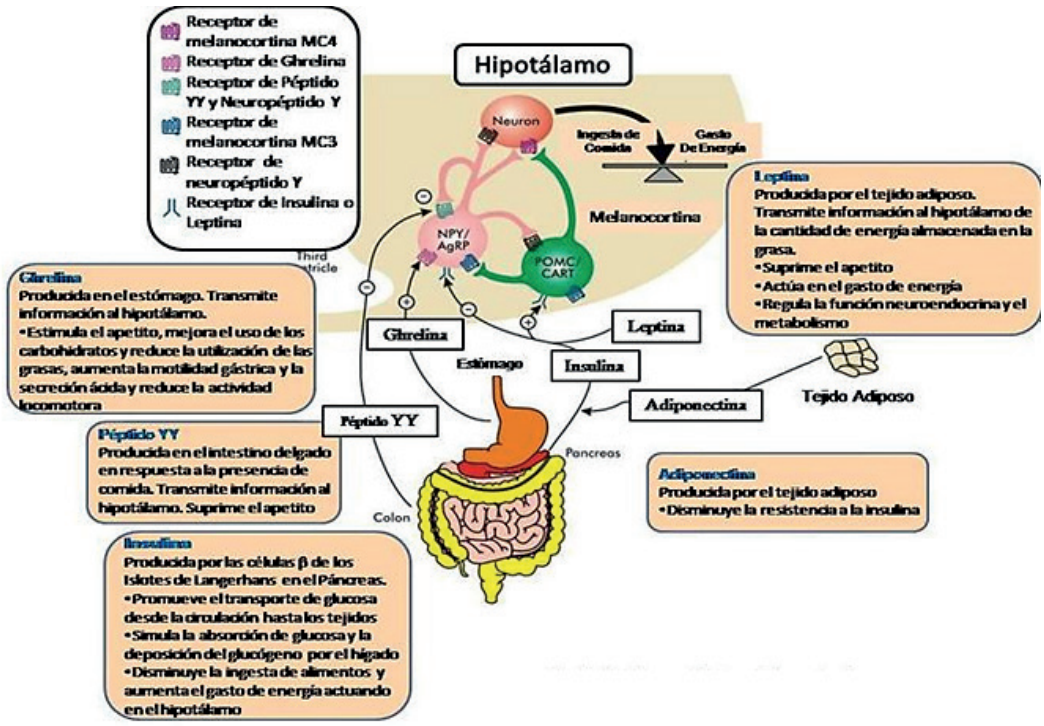
El carácter rústico de la raza ibérica es el resultado de su adaptación al bosque mediterráneo en el que vive: la dehesa. Su capa oscura, su capacidad de locomoción y su gran capacidad de ingesta le permiten conseguir un adecuado aprovechamiento de los recursos naturales de la dehesa (bellotas y pasto), con buena adaptación a lo estacional de la disponibilidad de alimentos y a las condiciones climáticas extremas. El cerdo ibérico se ha adaptado durante siglos a las duras condiciones ambientales del SO de la Península Ibérica, mediante diversos mecanismos genéticos, uno de los cuales es el desarrollo de un genotipo ahorrador. Este genotipo, descrito en otras especies de mamíferos y causante de fenotipos obesos, permite acumular energía en forma de abundantes depósitos grasos durante épocas de disponibilidad de alimento para sobrevivir en periodos de escasez y clima adverso (Neel, 1962).

Su marcado carácter adipogénico es uno de los factores más influyentes en la calidad de su productos (López-Bote, 1998). Las características de su grasa

intramuscular y subcutánea son determinantes en la obtención de productos de alta calidad sensorial.

Es también importante resaltar su mayor capacidad de ingestión voluntaria con respecto a otras razas magras, pues contribuye notablemente a la cantidad de grasa corporal depositada (Morales *et al*., 2002). Estudios recientes basados en secuencias genómicas han identificado huellas de selección en el genoma ibérico relacionadas con cambios en la ingesta de alimentos (Esteve-Codina *et al*., 2013). Uno de los principales mecanismos que intervienen en la regulación fisiológica del apetito es el nivel plasmático de leptina.

Figura 5: Regulación hipotalámica del apetito (Fuente: Diagnostic Systems Laboratories. Inc)



La leptina es una hormona periférica reguladora de la saciedad producida principalmente por los adipocitos en función de la cantidad de materia grasa. Es liberada al torrente sanguíneo en respuesta al estado nutricional, especialmente a los niveles de insulina y glucocorticoides, llegando a su receptor específico (LEPR) localizado principalmente en el hipotálamo y produciendo su activación y desencadenando una cascada de señalización que produce un ajuste del balance energético con aumento de sensación de saciedad (Figura 5). La adiponectina es otra hormona que aumenta la sensibilidad a la insulina

participando en el metabolismo de la glucosa y los ácidos grasos. La insulina es secretada por las células pancreáticas en función del nivel de glucosa y es el principal activador del almacenamiento energético en el tejido adiposo (Keiffer and Habener, 2000).

El cerdo ibérico presenta niveles plasmáticos de leptina muy superiores a los encontrados en el suero de cerdos magros (Fernandez-Figares *et al*., 2007), existiendo una correlación positiva entre el nivel de leptina en plasma y el espesor de tocino dorsal (Deng *et al*., 2007). Ademas, se ha descrito una variante estructural en el gen del receptor de leptina (LEPR), con un alelo (LEPR c.1987T), fijado en la raza ibérica y que en diferentes entornos genéticos ha mostrado efectos sobre el apetito, crecimiento tardío y el engrasamiento (Óvilo *et al*., 2005; Canovas *et al*., 2009; Rodríguez *et al*., 2010; Estany *et al*., 2014) y que influye en la regulación transcripcional a nivel hipotalámico del balance energético (Óvilo *et al*., 2010). Los altos niveles plasmáticos de leptina, junto con el mayor apetito y engrasamiento observados en la raza ibérica, son características compatibles en medicina humana con el síndrome de resistencia a la leptina (Myers *et al*., 2008) es por esto, que el cerdo ibérico puede ser utilizado como modelo animal para estudios de algunos tipos de obesidad y síndromes asociados. Además el cerdo como especie modelo proporciona un mayor conocimiento de la progresión de enfermedades y nuevos tratamientos potenciales pues refleja mejor que otras especies modelo las enfermedades humanas dado su mayor semejanza anatómica, fisiológica y genética (Cabot *et al*., 2001; Walters *et al*., 2012).

Estas particularidades en su metabolismo favorecen una mayor acumulación de tejido adiposo subcutáneo y una mayor infiltración de grasa en el músculo y contribuyen a la diferenciación de su perfil metabólico respecto a otras razas mejoradas. Por ejemplo, en una comparación entre cerdo ibérico y Landrace, el espesor del tocino dorsal fue de 48.1 mm en ibéricos frente a 20.7 mm en los cerdos Landrace (Serra *et al*., 1998). En el mismo estudio, los cerdos ibéricos mostraron un contenido medio en grasa intramuscular (GIM) de 3.91%, mientras que dicho valor fue de 0.66% en los cerdos Landrace.

Además la raza ibérica posee un característico perfil de ácidos grasos (AG) que refleja claramente la composición de AG presente en las bellotas y en el pasto (Rey *et al*., 2006), con un mayor contenido de ácidos grasos monoinsaturados (AGMI), principalmente oleico, y menor de ácidos grasos saturados (AGS) que

los cerdos producidos en sistemas intensivos (Cava *et al*., 1997; Tejerina *et al*., 2012). Además de la influencia de la alimentación en los sistemas extensivos, los cerdos ibéricos tienen una capacidad de desaturación de grasa superior a otras razas magras (Barea *et al.,* 2013). Por ejemplo, cuando se someten a una misma dieta, los cerdos Landrace x Large White presentan un 44% de ácido oleico frente a 45.6% (p<0.01) en ibérico en grasa subcutánea dorsal (Barea *et al*., 2013) y cerdos Landrace presentan un 46.5% frente a 48.9% (p<0.001) de ácido oleico en ibérico, también en grasa subcutánea (Serra *et al*., 1998).

El cerdo ibérico se caracteriza además por una acumulación de proteína comparativamente menor a la observada en otras razas porcinas seleccionadas (Barea *et al*., 2007). Una elevada tasa de degradación proteica puede ser la causa principal del menor desarrollo muscular de los cerdos ibéricos de raza pura (Óvilo *et al.*, 2014b). Existen diversos estudios que proporcionan información molecular sobre los mecanismos potenciales que explicarían el fenotipo de crecimiento y engorde singular de los cerdos ibéricos, consistente en una diferenciación e hipertrofia de adipocitos más temprana y menor tasa de deposición de proteínas en sus músculos (Óvilo *et al*., 2014b; Ayuso *et al*., 2015b).

Todas estas peculiares características, proporcionan a su carne una textura, aroma y sabor únicos y les confieren un alto valor nutritivo y económico. Como contrapartida, la raza ibérica presenta un peor desarrollo muscular y requiere un largo tiempo para obtener sus productos, por lo que es habitual el cruzamiento con la raza duroc, también de capa oscura, para mejorar su rendimiento productivo. El cruce con esta raza supone una mejora en la prolificidad, la tasa de crecimiento y el contenido magro (López-Bote, 1998).

La raza duroc es una raza procedente de EEUU, que surge por fusión de la Old Duroc (New York) y la Red Jersey (New Jersey) en el siglo XIX. Estas dos razas fundadoras, a su vez, proceden de cerdos de muy distintos orígenes, incluyendo cerdos europeos como Berkshire, cerdos africanos con influencia ibérica como Colorado de Guinea, y muy posiblemente cerdos ibéricos de capa retinta. Desde el comienzo de la llegada de esclavos a Norteamérica en el siglo XVIII, existen datos de llegada de cerdos colorados desde Guinea y la costa oeste de África. Otro de los lugares de procedencia es Europa principalmente desde España distribuyéndose por Kentucky y la zona sur en 1837 y desde Portugal en 1852 y distribuida por Massachusetts, New York, Vermont y otros estados (Vaughan,

1950). En la actualidad se trata de una raza cosmopolita, con líneas seleccionadas con distintos propósitos en muchos países. Esta raza se introdujo en España en la década de los sesenta, con la denominación de Duroc-Jersey, y actualmente se está utilizando también como raza finalizadora en los cruces industriales de porcino blanco, ya que proporciona una infiltración grasa que hace que mejoren sus productos en cualidades y sabor.

El cruce de cerdo ibérico está aceptado exclusivamente con cerdos de la raza duroc. Un elevado porcentaje del total de cerdos sacrificados bajo la Norma de calidad de los productos del ibérico (Real Decreto 4/2014 modificado por el Real Decreto 255/2016) corresponde a animales ibéricos cruzados al 50% o 75% con duroc. Para asegurar el mantenimiento de la variabilidad genética y de la población de raza ibérica, la genética duroc sólo puede ser utilizada como línea paterna terminal.

Sin embargo, se ha descrito un impacto negativo de la introducción de genética duroc sobre la calidad de los productos ibéricos en diferentes publicaciones científicas. Algunos de los factores que pueden verse alterados por el empleo de genética duroc son una disminución de la GIM y de los AGMI en los animales cruzados (Ventanas *et al*., 2006; Fuentes *et al*., 2014). Éste es sin duda, el efecto más importante desde el punto de vista de la calidad de los productos ibéricos. Otros efectos se refieren a las características organolépticas. En cuanto al sabor, los jamones ibéricos se consideran ligeramente más amargos (Carrapiso *et al*., 2003). Los lomos curados de animales puros son más jugosos (Ventanas *et al.,* 2007) que los procedentes de animales cruzados y también el veteado es mayor en jamones y lomos curados ibéricos (Carrapiso *et al*., 2003; Ventanas *et al*., 2007). Respecto al color, los jamones ibéricos muestran mayor predominio del color rojo en fresco, aunque esta diferencia no se mantiene tras el curado (Andrés *et al*., 2001) y respecto al brillo también es mayor en lomos curados de cerdos ibéricos puros (Andrés *et al*., 2001; Ventanas *et al*., 2007). También cambia el perfil de los triglicéridos presentes en la grasa que es más insaturado, promoviendo la oxidación de los lípidos, afectando a rasgos sensoriales como la apariencia al corte y la sequedad de la carne (Tejeda *et al*., 2002; Petrón *et al.,* 2004).

Estas diferencias en la composición y en las características del músculo y de la grasa entre razas están determinadas por factores genéticos. Entre ellos se

conocen algunas mutaciones causales como la mutación descrita por Van Laere y colaboradores (2003) en el gen *IGF2*, un gen candidato relacionado con el crecimiento muscular y con la calidad de la carne y que se halla prácticamente fijado en las líneas duroc actuales (Jeon *et al*., 1999; Nezer *et al*., 1999). Sin embargo, el conocimiento sobre los mecanismos moleculares implicados en las diferencias en calidad de carne y grasa entre cerdos ibéricos y duroc puros es todavía muy limitado.

1.3.1. SISTEMAS PRODUCTIVOS EN LA RAZA IBERICA

El sistema de producción tradicional de cerdo ibérico se basa en el sacrificio a pesos elevados (mínimo 108 y 115 kg de peso de la canal para ibéricos puros y cruzados, respectivamente) y con una edad mínima de 10 a 14 meses, debido a que son destinados principalmente a la elaboración de productos curados. La consistencia de la carne aumenta con la edad por un sobrecruzamiento progresivo del colágeno muscular (Mayoral *et al*., 1999). Probablemente este factor (sin duda también influido por el ejercicio en el periodo de montanera en la dehesa) sea importante en el mantenimiento de la textura de los productos cárnicos del ibérico, especialmente teniendo en cuenta que la grasa del cerdo ibérico presenta un alto grado de insaturación, lo que disminuye su consistencia. Por otra parte, el contenido en grasa, incluyendo la de infiltración, aumenta tanto con el peso como con la edad de sacrificio, mejorando de esta forma las características organolépticas del producto elaborado.

Las principales razones de la alta calidad de sus productos elaborados es la gran calidad de la materia prima y el cuidado puesto en el proceso de curado y conservación. La duración de este proceso puede variar, pero el Real Decreto 4/2014, modificado por el Real Decreto 255/2016, exige un mínimo de 600 días de curación para jamones de menos peso y de 730 días para jamones más pesados. Esto favorece el desarrollo del aroma, sabor y otras características óptimas del producto final. En relación a las características de la materia prima, el contenido en GIM, así como su composición (en concreto la cantidad de ácido oleico), son factores determinantes en la calidad de los productos ibéricos (Ventanas *et al*., 2005).

Los principales productos curados que se obtienen del cerdo ibérico son: jamón, paleta, lomo y embutidos curados. Además, los productos procedentes de

animales ibéricos se enmarcan dentro de la norma de calidad para la carne, el jamón, la paleta y la caña de lomo ibérico (Real Decreto 4/2014 modificado por el Real Decreto 255/2016), que establece las siguientes denominaciones:

a) Designación por alimentación y manejo:
   — De bellota.
   — De cebo de campo.
   — De cebo.

b) Designación por tipo racial:
   — 100% ibérico.
   — 75% ibérico.
   — 50% ibérico.

La mayoría de la producción se englobaría dentro de los animales de cebo intensivo y 50% ibérico según las diferentes denominaciones de la norma de calidad. Sin embargo, se considera de mayor relevancia la producción de animales 100% ibérico y de bellota, por la excelencia en la calidad y valor económico de sus productos, aunque tengan una producción mucho menor, como se desprende del censo de animales de cada designación correspondiente al año 2017 (Tabla 1). Es este uno de los motivos por los que en esta tesis y en sus ensayos se hayan utilizado animales ibéricos 100%

Tabla 1. Censo de animales ibéricos del año 2017 según la norma de calidad. Fuente RIBER (Registro informativo de ibérico, MAPAMA).

CENSO DE ANIMALES (España y Portugal) 2017										
RD 4/2014 Informe generado el 21/09/2018										
Año	Trimestres	Ibérico 100% Bellota	Ibérico 100% Cebo Campo	Ibérico 100% Cebo	Ibérico 75% Bellota	Ibérico 75% Cebo Campo	Ibérico 75% Cebo	Ibérico 50% Bellota	Ibérico 50% Cebo Campo	Ibérico 50% Cebo
2017	1 trimestre	299178	13034	10923	121906	20913	4643	261923	136020	463585
	2 trimestre	52	15811	9708	100	20438	5406	0	172620	474036
	3 trimestre	0	5442	6875	38	7517	4500	0	110156	338869
	4 trimestre	7520	13147	14130	1216	8366	5900	2507	140178	583889
	TOTAL	306750	47434	41636	123260	57234	20449	264430	558974	1860379

1.4. LOS DEPOSITOS GRASOS EN EL CERDO

Entre los depósitos grasos animales, podemos diferenciar tres grandes tipos: la grasa subcutánea o de cobertura (60-70% del total), la grasa visceral (10-15%) y la grasa inter e intramuscular (20-30%), siendo estas últimas las que

se depositan, respectivamente, entre los paquetes musculares o entre y dentro de las fibras musculares, en forma de membranas lipídicas y pequeñas vacuolas grasas (Gerbens, 2004). Desde un punto de vista productivo los depósitos grasos más relevantes son la grasa subcutánea por ser el depósito más extenso y la grasa intramuscular por su papel en la calidad de la carne. El tejido graso subcutáneo, además de ser cuantitativamente el más importante, manifiesta un crecimiento más rápido que otros depósitos, y a su vez comprende el tejido adiposo subcutáneo dorsal o tocino y el tejido adiposo subcutáneo abdominal o panceta.

1.4.1. LA ADIPOGÉNESIS Y SU REGULACIÓN

La adipogénesis es el proceso de diferenciación de células grasas precursoras (preadipocitos) hacia células grasas maduras (adipocitos). Los adipocitos son las células mayoritarias en el tejido adiposo y se clasifican en tres tipos: adipocitos pardos, adipocitos blancos y adipocitos "brite" (adipocitos beige) (Petrovic *et al*, 2010).

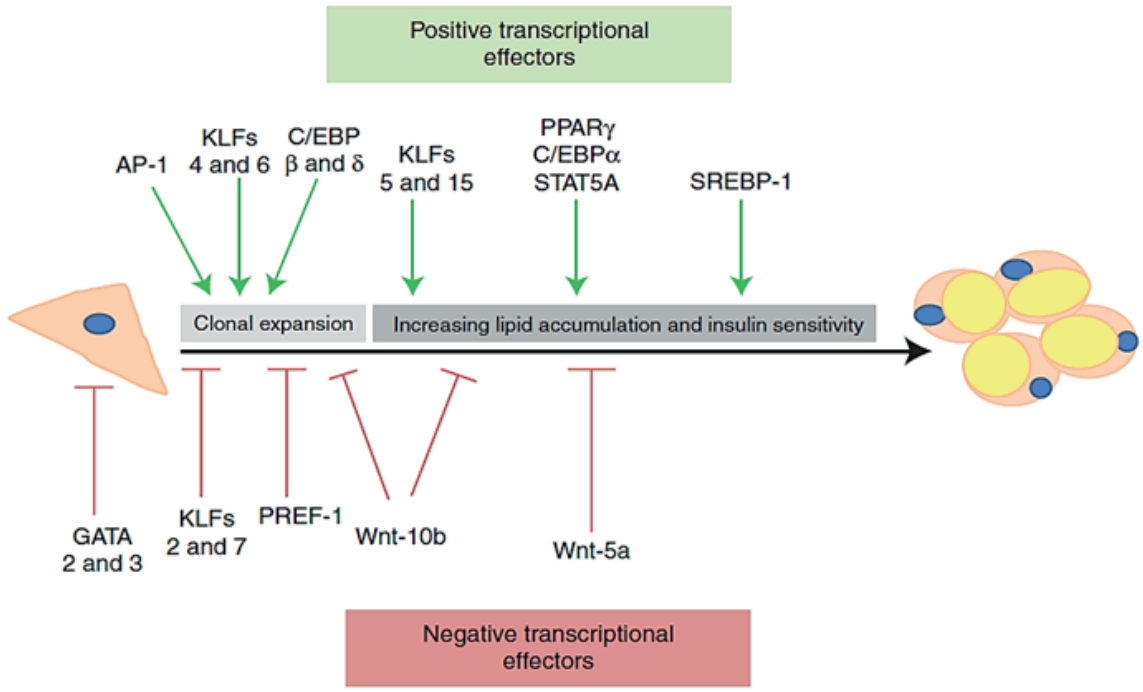
La diferenciación celular es el proceso por el cual las células se vuelven estructural y funcionalmente distintas entre sí y se convierten en tipos celulares definidos (Wolpert *et al*., 2015). Atendiendo al grado de diferenciación, dentro de cualquier organismo podemos encontrar células de tres tipos: células madre (las más indiferenciadas), células precursoras y células diferenciadas. Todas ellas son descendientes de células madre embrionarias que, tras sucesivas divisiones van aumentando en su grado de especialización (Lodish *et al*., 2000).

En porcino, la detección de adipocitos comienza al inicio del último tercio de gestación (Hausman y Thomas, 1986) y la mayor expansión del tejido adiposo ocurre rápidamente tras el nacimiento. Sin embargo, el desarrollo del tejido adiposo es un proceso continuo a lo largo de la vida y depende, aparte de factores genéticos, de factores ambientales. Además, una vez que estas células se diferencian el proceso es irreversible (Ailhaud y Hauner, 1998). Es por ello de gran importancia conocer cómo se produce y regula la adipogénesis en el cerdo con el fin de encontrar mecanismos para modificar la cantidad y la composición de los depósitos grasos.

Los preadipocitos y adipocitos encontrados en la grasa subcutánea e intramuscular muestran diferencias importantes en cuanto a los procesos de adipogénesis y lipogénesis. En primer lugar, los preadipocitos musculares comienzan el proce-

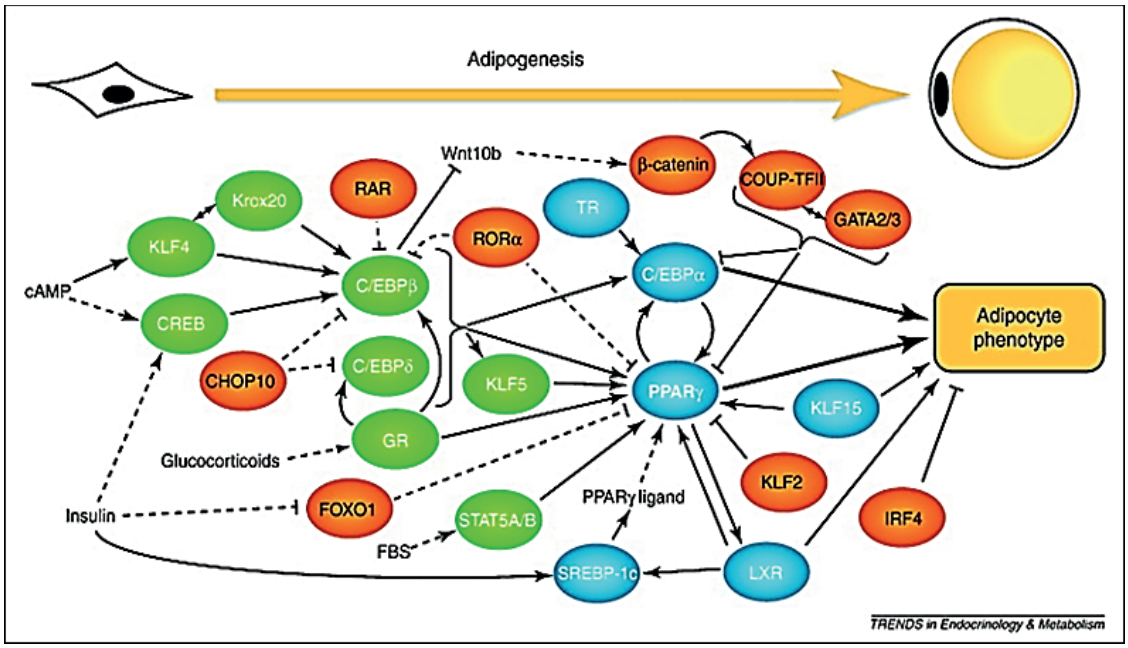
so de diferenciación de una forma más tardía que los de la grasa subcutánea, tanto en cultivos celulares, tratados con inductores de la diferenciación (Wang *et al*., 2013), como *in vivo* (Gondret *et al*., 2008). Más allá de las diferencias relativas a la diferenciación adipocitaria, también se han encontrado diferencias en el metabolismo lipídico entre adipocitos musculares y de la grasa subcutánea. Así, se ha observado que los adipocitos musculares tienen preferencia por el uso de la glucosa para la síntesis lipídica, mientras que los adipocitos de la grasa subcutánea utilizan principalmente ácidos grasos durante la lipogénesis (Wang *et al*., 2013). Por último, se ha descrito que los adipocitos localizados en el depósito subcutáneo acumulan más lípidos y de forma más rápida que los adipocitos musculares (Zhou *et al*., 2010; Kouba y Mourot, 2011). Son numerosos los mecanismos celulares y moleculares que controlan el proceso de diferenciación adipocitaria mediados por la activación o represión de distintos factores de transcripción (Ma *et al*., 2015). Dentro de los factores de transcripción involucrados en la regulación de la adipogénesis, la familia CEBP (CCAAT-enhancer binding proteins) y la familia PPAR (peroxisome proliferator-activated receptors) son cruciales y los que más ampliamente han sido estudiados (Rosen y MacDougald, 2006; Sarjeant and M.Stephens, 2012). (Figura 6).

Figura 6. Factores de transcripción involucrados en el desarrollo de los adipocitos (Sarjeant and Stephens, 2012).



La familia CEBP está constituida por varias isoformas, entre ellas, CEBPA, CEBPB, CEBPG, CEBPD y CHOP se expresan en los adipocitos. La activación o inhibición de estos genes sigue una secuencia temporal en la que la expresión temprana de CEBPB y CEBPD conduce a la expresión, ya en la fase tardía de CEBPA y PPARG (Rosen y MacDougald, 2006) (Figura 7).

Figura 7. Genes involucrados en la cascada de activación de la adipogénesis. (Fuente: Siersbæk et al., 2011)



CEBPA parece ser un factor nuclear indispensable y crítico en el proceso de diferenciación de los adipocitos (Yeh *et al*., 1995) que induce la expresión de forma directa de numerosos genes específicos del adipocito maduro, en los cuales se han identificado lugares de unión a CEBPA en las regiones promotoras (MacDougald y Lane, 1995). Además, sus efectos van más allá de lo observado en cultivos celulares, puesto que ratones con este gen reprimido (CEBPA-/-) carecen totalmente de tejido adiposo (excepto en la glándula mamaria) (Linhart *et al*., 2001). Varios estudios han puesto de manifiesto que este factor de transcripción es necesario y suficiente, para poner en marcha el proceso de diferenciación de los adipocitos incluso en ausencia de agentes inductores de la diferenciación (Freytag *et al*., 1994). En concordancia, se ha observado que la supresión de la expresión de CEBPA en cultivo celular provoca una inhibición en la diferenciación terminal de los adipocitos, lo cual parece indicar que este

proceso requiere el mantenimiento de la expresión sostenida de CEBPA (Lin y Lane, 1992).

A pesar de la importancia de la familia CEBP en la regulación de la adipogénesis, estos factores de transcripción son claramente insuficientes para activar el proceso de diferenciación sin la presencia de PPARG, el cual ha sido relacionado con la adipogénesis como uno de los factores de transcripción cruciales en el desarrollo de los adipocitos tanto *in vitro* como *in vivo* y es considerado como el "director" de la adipogénesis (Tontonoz *et al*., 1994). Es el único miembro de una familia de receptores nucleares/factores de transcripción PPAR que se encuentra expresado en altos niveles específicamente en tejido adiposo. Además de la regulación transcripcional, algunos estudios relacionan la expresión de PPARG con los niveles de lípidos en el organismo, destacando su función en el mantenimiento de la homeostasis lipídica (Morrison y Farmer, 2000) y no sólo como regulador de la adipogénesis.

Otro factor que también parece estar implicado en el proceso de diferenciación es SREBP1C, que es uno de los tránscritos codificados por el gen *SREBF1*. La coexpresión de este factor de transcripción incrementa la actividad transcripcional de PPARG incluso en ausencia de sus ligandos activadores (Kim y Spiegelman, 1996). SREBP1C es el factor de transcripción que presenta más inconsistencias entre estudios *in vivo* e *in vitro*. Curiosamente, estudios *in vivo* con ratones SREBP1C-/- han demostrado que éstos desarrollan todos los depósitos grasos con normalidad (Shimano *et al*., 1997).

El papel de los receptores nucleares LXR en el control de la adipogénesis es controvertido, puesto que diferentes estudios han revelado efectos positivos, negativos y neutros. Sin embargo, su papel en la regulación de la fisiología del adipocito maduro sí está más clara (Rosen y MacDougald, 2006).

El proceso de adipogenesis transforma el preadipocito en una célula esférica que comienza a acumular lípidos (hipertrofia) y va adquiriendo las características morfológicas y funcionales de un adipocito maduro, después se detiene el crecimiento celular y se da la expansión clonal (Gregoire *et al*., 1998) indispensable para la diferenciación terminal del adipocito. Tras la diferenciación terminal se inicia la activación de genes específicos del adipocito maduro. Así, se produce un incremento de la expresión y actividad de enzimas lipogénicas como FASN, ME1, G3PDH, ACACA o SCD1 (Spiegelman *et al*., 1993). Durante esta etapa aumenta

también la sensibilidad a la insulina, debido al aumento en el número de receptores de insulina y transportadores de glucosa dependientes de insulina (GLUT4) (Moreno-Aliaga y Martinez, 2002). Además de la expresión de genes relacionados con el metabolismo lipídico, las células también sintetizan otros productos considerados específicos del tejido adiposo, como aP2 (una proteína fijadora de AG específica de adipocitos) o perilipina, (proteína asociada a las gotas lipídicas), así como sustancias endocrinas y paracrinas (como por ejemplo leptina, adiponeptina y monobutirina) (Gregoire *et al*., 1998) hormonas que como se ha mencionado anteriormente tiene un papel primordial en la regulación del peso corporal y en la ingesta de alimentos.

Por tanto, el adipocito maduro es una célula que no solo se encarga de almacenar lípidos como forma de energía sino que también sintetiza un gran número de factores de crecimiento, citoquinas y hormonas que participan en la homeostasis de la energía en general y más específicamente en la homeostasis de los lípidos y en la modulación de la respuesta inflamatoria (Ahima *et al*., 2006; Ferrante, 2007; Gutierrez *et al*., 2009; Lee and Lee., 2014).

1.4.2. FACTORES QUE INFLUYEN EN EL CONTENIDO DE LOS DEPOSITOS GRASOS

Existen diversos factores capaces de modificar la cantidad y características de los depósitos grasos del animal y que pueden ser intrínsecos como el genotipo, el sexo, la edad o estado embrionario o factores extrínsecos como el sistema de producción, especialmente el nivel de ingesta y el tipo de alimentación.

1.4.2.1. Genética

La raza, y por lo tanto la genética, ha sido destacada como la principal causa que determina la cantidad de grasa en el cerdo, con grandes diferencias entre razas e incluso entre variedades o estirpes (Muriel *et al*., 2004). Entre las diferentes razas porcinas, existen líneas de elevado potencial de crecimiento proteico y otras cuyo crecimiento magro es más limitado siendo estas últimas líneas más grasas, con crecimientos más lentos, peores índices de conversión, mayor tamaño de los adipocitos y hasta el doble de espesor de la grasa subcutánea dorsal que las líneas magras, como es el caso de la raza ibérica (López-Bote, 1998; Serra *et al*., 1998). Las razas porcinas actuales han sido seleccionadas para obtener

una carne más magra al gusto de los consumidores, ya que se le atribuyen características más saludables (Lonergan *et al*., 2001; Wood *et al*., 2008). En consecuencia, esta selección ha producido un descenso marcado en el contenido de GIM en las líneas modernas. Por el contrario, en razas menos seleccionadas este parámetro es muy variable, pudiéndose encontrar desde razas poco engrasadas, como el tamworth, hasta razas con un alto nivel de infiltración grasa, como es el caso del cerdo ibérico.

Para valorar la contribución de la variación genética o de la variación intrapoblacional sobre un carácter hay que tener en cuenta la heredabilidad de dicho carácter, que es la proporción de su varianza que viene determinada por la variación de los efectos genéticos aditivos. En porcino, los caracteres de composición corporal presentan una heredabilidad media o alta que facilita la respuesta a la selección de los mismos. Así por ejemplo en cerdo ibérico los valores de la heredabilidad del porcentaje de GIM en *l. dorsi* estimados en distintas líneas y sistemas de manejo oscilan entre 0.25 y 0.66 (Fernández *et al*., 2003; García-Casco *et al*., 2014; Ibáñez-Escriche *et al*., 2016; Muñoz *et al*., 2018). Estos estudios muestran que la selección convencional para este carácter está dificultada por varios factores: a) registro tardío en animales sacrificados emparentados con los candidatos a la selección; b) antagonismo genético con caracteres de rendimiento cárnico y c) interacción con el sistema de producción. Por todo ello parece aconsejable la utilización en la selección de información genómica (GWAS o basada en alelos favorables de genes candidatos).

El contenido en grasa está regulado por numerosos genes y rutas metabólicas. Algunos de los genes candidato que podrían estar relacionados son:

a) Genes candidatos relacionados con el desarrollo adipocitario:
   Son, principalmente, factores de transcripción que regulan el desarrollo y la diferenciación de adipocitos, algunos mencionados anteriormente: *PPARG, RXRG, RARA, CEBPA, CEBPB, ASXL2, DLK1, EGR2, KLF5* (Tontonoz *et al*., 1994; Darlington *et al*., 1998; Rosen *et al*., 2002; Chen *et al*., 2005; Oishi *et al*., 2005; Park *et al*., 2011).
b) Genes candidatos relacionados con la síntesis y metabolismo de ácidos grasos:
   Se expresan en el adipocito maduro, su expresión regula los procesos de lipogénesis y lipolisis y por lo tanto, influye sobre el contenido lipídico y el volumen de los adipocitos: *SREBP1F, ACACA, FASN, SCD1, ME1, ELOVL6,*

FABP4, DGAT, CPT1, AdPLA, ACSL4, ACOX1, ATGL, HSL, MGLL (Singh *et al*., 1992; Nechtelberger *et al*., 2001; Cronan y Waldrop, 2002; Horton *et al*., 2002; de Sousa *et al*., 2005; Damon *et al*., 2006; Durgan *et al*., 2006; Kim *et al*., 2011; Corominas *et al*., 2013b; Skiba *et al*., 2013; Tuohetahuntila *et al*., 2015; Lass *et al*., 2011; Zechner *et al.*, 2012).

c) Genes candidatos relacionados con el control de la homeostasis energética y la ingesta voluntaria y que se expresan principalmente en el hipotálamo: Son neurotransmisores y receptores que en respuesta a señales periféricas dan lugar a cascadas de señalización que modulan la ingesta y el gasto energético (Óvilo *et al*., 2010). Entre los genes regulados por estas cascadas de señalización cabe destacar: *LEPR*, *MC4R, POMC, NPY, AGRP* y *CART* (Kristensen *et al*., 1998; Elias *et al*.,1999; Korner *et al*., 2001; Balthasar *et al*., 2004) entre otros.

d) Genes candidatos relacionados con el metabolismo de la glucosa:
La regulación del metabolismo de la glucosa tiene también una importante relación con el control de la homeostasis energética (Könner *et al*., 2009). Algunos de los genes relacionados con este proceso son: *IGF1, INS, INSR, INSIG1, GLUT, G6PC, PCK1, PCK2* (Garcia de Herreros y Birnbaum, 1989; Ramsay *et al*., 1989; Beale *et al*., 2007; Liu *et al*., 2008; Könner *et al*., 2009 Latorre *et al*., 2016).

1.4.2.2. Sexo/Castración

En los sistemas de producción tradicionales, tanto los machos como las hembras son castrados, en los machos para evitar el olor sexual del verraco en los productos cárnicos y en las hembras para evitar la pérdida de rendimiento que ocurre durante el estro. En cuanto a la influencia del sexo o la castración, hay resultados contradictorios.

En los trabajos realizados en la especie porcina sobre el efecto del sexo o la castración en el contenido en grasa, algunos de ellos (Latorre *et al*., 2003; Correa *et al*., 2006) reportan un efecto significativo del sexo (por ejemplo mayor porcentaje de GIM en machos castrados que en hembras) mientras que otros (Cisneros *et al*., 1996b; Hamilton *et al*., 2000; Latorre *et al*., 2004) no detectan ningún efecto. También se ha observado una menor cantidad de AGPI en machos castrados con respecto a las hembras en cerdo ibérico (Serrano *et al*., 2009).

1.4.2.3. Edad o estado de desarrollo

El porcentaje de grasa corporal del animal aumenta conforme lo hace su peso. Así, desde el nacimiento hasta el sacrificio el crecimiento del tejido graso aumenta y lo hace de forma superior al crecimiento del tejido magro de la canal.

Los lechones nada más nacer no tienen capacidad de sintetizar grasa a partir de carbohidratos, por lo que el engrasamiento del animal en estas primeras etapas de su vida tiene lugar mediante el depósito directo de la grasa contenida en la leche materna. Al crecer el animal se van activando las enzimas responsables de la lipogénesis (Lakshmanan *et al*., 1972).

El desarrollo celular del tejido adiposo tiene lugar en tres fases: la hiperplasia (entre los 7 y 20 kg de peso vivo), hiperplasia e hipertrofia conjunta (entre los 20 y 70 kg de peso vivo) y la hipertrofia dominante (a partir de los 70 kg de peso vivo) (Hauser *et al*., 1997). Por tanto, el incremento de los depósitos grasos durante el crecimiento y cebo es la principal consecuencia del llenado del adipocito o hipertrofia, existiendo una correlación positiva entre el peso vivo del animal y la evolución del diámetro de los adipocitos de los diferentes depósitos grasos.

En general, existe una relación entre la edad al sacrificio y la cantidad de grasa, ya que es en estadios más tardíos del desarrollo cuando los depósitos grasos aumentan. Este hecho, aunque está bien documentado (Gerbens, 2004; Bosch *et al*., 2012), no siempre ha sido observado en trabajos previos (Ellis *et al*., 1996; Lo Fiego *et al*., 2010).

1.4.2.4. Sistema de producción

El sistema de producción y la alimentación son los factores extrínsecos al animal que determinan en última instancia la síntesis de grasa. Por ello, se han estudiado distintas estrategias nutricionales para modificar este contenido graso aunque existen muchos mecanismos aún no bien conocidos. El sistema de producción engloba a su vez distintos parámetros, como son el ambiente, el ejercicio y la alimentación (Cava *et al*., 1999; Edwards, 2005; Rey *et al*., 2006a; López-Bote *et al*., 2008). El sistema más extendido en producción porcina es el sistema de cebo intensivo. En el caso del porcino ibérico, es más complejo, puesto que hay distintos sistemas productivos aceptados (según la norma de calidad

Real Decreto 255/2016), mencionados con anterioridad. El sistema de producción ha demostrado tener efecto sobre el perfil de AG de los depósitos grasos, así, se ha observado que los cerdos alimentados en montanera presentan un perfil más rico en C18:1 n-9 (oleico), C18:3 n-3 (linolénico) y AGMI, con un menor contenido en AGS que los alimentados en intensivo (Cava *et al*., 1997; Ruiz *et al*., 1998; Andrés *et al*., 2001; Tejerina *et al*., 2012). Este perfil refleja la composición de ácidos grasos presente en las bellotas, junto con el alto contenido en C18:3 n-3 de la hierba (Rey y López-Bote, 2001; Rey *et al*., 2006b). Por otro lado, los animales de recebo presentan valores intermedios (Ruiz *et al*., 1998; Andrés *et al*., 2001; Tejerina *et al*., 2012). Los resultados obtenidos al evaluar el contenido en grasa son variables, encontrando trabajos que describen un efecto positivo de la producción en montanera sobre el contenido de grasa (Andrés *et al*., 2001; Ventanas *et al*., 2007) mientras que en otros casos, los resultados no muestran diferencias significativas (Carrapiso y García, 2005; Tejerina *et al*., 2012).

Pese a que la alimentación forma parte del sistema de producción, debido a que tiene un papel decisivo en la deposición grasa será explicado de forma independiente más adelante.

1.5. LOS ÁCIDOS GRASOS Y SU METABOLISMO

El origen de los lípidos acumulados en los depósitos grasos del cerdo es el resultado de un balance entre la deposición directa de los AG procedentes de la dieta y la síntesis endógena de estos o lipogénesis *de novo.* Existen diferencias entre las localizaciones anatómicas donde se depositan estos lípidos. Hay áreas como el cuello donde se deposita fundamentalmente la grasa proveniente del alimento y otras en las que la síntesis endógena adquiere mayor relevancia como es el caso de la grasa subcutánea (Mourot *et al*., 1995 y 1996).

También existen diferencias entre especies y entre razas en la composición de ácidos grasos de los distintos depósitos grasos. En el tejido adiposo porcino, la composición en AG de los triglicéridos tiene una mayor cantidad de ácido oleico y palmitoleico y una menor cantidad de esteárico en comparación con otras especies (Wood et al., 2004). Esta composición puede verse influida por la raza y en concreto los cerdos ibéricos poseen un porcentaje mayor de ácido oleico y palmitoleico que el cerdo blanco. A nivel del hígado, la composición en AG de los triglicéridos presentan una mayor concentración de mirístico, palmítico, palmitoleico

y araquidónico (en menor concentración en el tejido subcutáneo) (Miller et al., 1990) y una menor cantidad de esteárico, oleico, linoleico y linolénico comparado con otras especies. En el músculo, la proporción de triglicéridos con respecto a los lípidos totales oscila entre el 50 y el 68% (Lesigneur-Meyner y Gandemer, 1991), variando su composición en AG en el cerdo en función de ciertos factores como los indicados para el tejido adiposo. Por ejemplo, el cerdo ibérico, al ser una raza con mayor engrasamiento, posee un nivel de ácidos grasos insaturados menor que otras razas más magras (puesto que al tener mayor cantidad de lípidos de reserva, el porcentaje de fosfolípidos o lípidos polares que son fundamentalmente estructurales (ricos en ácidos grasos insaturados) disminuye. Respecto a los fosfolípidos o lípidos polares, se caracterizan por tener carácter anfipático (son hidrofóbicos e hidrofílicos) lo que les permite formar parte de las membranas celulares. En el músculo los fosfolípidos representan entre 16 al 32% de los lípidos totales (Flores y Nieto, 1985), encontrándose en menor cantidad en el tejido adiposo y apareciendo en mayor porcentaje en el hígado (30-40%).

1.5.1. LA DEPOSICIÓN DIRECTA

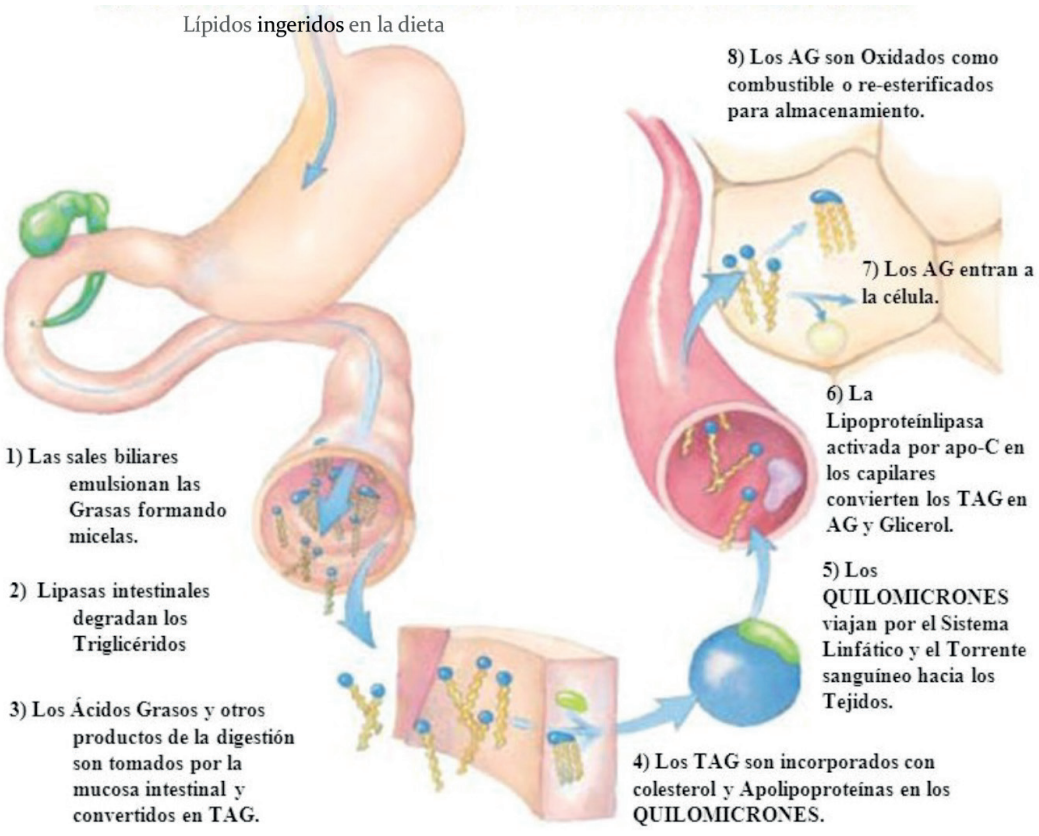
La deposición directa de los AG de la dieta se lleva a cabo por la digestión, absorción y transporte con muy pocas modificaciones en este proceso (Enser *et al.*, 2000). Son dos los factores principales que afectan a la asimilación global de la grasa de la dieta: la alimentación (composición de la grasa y régimen que reciba el animal) y la edad. Cuando los animales reciben la alimentación *ad libitum* reciben un exceso de aporte de energía sobre todo en forma de carbohidratos que favorece el depósito de AG procedentes de la síntesis endógena (AGS y AGMI), pero si la alimentación es restringida la síntesis de AG se ve limitada y cobra más importancia la deposición directa de AG (principalmente AGS). En cuanto a la edad, la asimilación de los AG de la dieta crece con la edad (tanto digestión como absorción) (Freeman, 1984).

Para la digestión y la absorción de los lípidos de la dieta (en su mayoría triglicéridos), primero es necesaria la solubilización, ya que son compuestos insolubles en el medio acuoso intestinal. Dicha solubilizacion sólo es posible por la incorporación a las micelas. Cuando la bilis se mezcla con las gotitas de lípidos en el intestino, los lípidos se absorben en las micelas y así se mantienen estables, pasando de formar parte de gotas de mayor tamaño, a micelas cuyo diámetro es mucho menor.

La digestión de los lípidos se lleva a cabo a nivel de intestino delgado gracias a la presencia de las enzimas lipolíticas del páncreas. La lipasa pancreática es la más importante y desdobla los triglicéridos (TG) de la dieta en a AG libres y monoglicéridos (Borgstrom, 1974).

Una vez producida la incorporación a las micelas ya pueden ponerse en contacto con las microvellosidades y absorberse a través de la membrana celular por difusión. Para penetrar en el interior de los enterocitos, las moléculas lipídicas difunden primero a la zona de líquido que rodea a éstos y luego penetran a través de la membrana epitelial. Las micelas difunden entonces en sentido retrógrado y vuelven a absorber nuevos lípidos, que son transportados hacia las células de las vellosidades (Figura 8).

Figura 8. Digestión, absorción y transporte de las grasas. Fuente: British Nutrition Foundation, 1992.



Tras penetrar en el enterocito, los AG libres y los monoglicéridos son captados por la proteína FABP (Fatty acid binding protein) que los transporta hasta el retículo endoplásmico liso donde son recombinados para formar nuevos triglicéridos (Jurie *et al*., 2007; Niot *et al*., 2009; Funaoka *et al*., 2010).

Diferentes estudios han evidenciado que no todos los AG se absorben con la misma intensidad, ya que la entrada de un lípido en fase micelar (paso necesario para su absorción) depende de su solubilidad en las sales biliares. Así los AG insaturados y de cadena media son más solubles y más capaces de aumentar la solubilidad micelar, y por tanto su absorción, que los AGS y de cadena larga (Freeman, 1984).

La mayor parte de los lípidos, ya sean sintetizados o ingeridos, pasan al torrente sanguíneo y son transportados a los diferentes tejidos donde son utilizados, ya sea como fuente de energía o como material estructural, incorporados a las membranas celulares como ocurre con los fosfolípidos (lípidos polares) o a los depósitos grasos del organismo en forma de triglicéridos (lípidos neutros). Los triglicéridos se forman al esterificarse los AG y el glicerol 3-fosfato.

La distribución de los triglicéridos plasmáticos está regulada por la diferente actividad entre los tejidos de la lipoprotein lipasa LPL, responsable de su hidrólisis en el endotelio vascular (Wood *et al*., 2008; Luo *et al*., 2009). En el tejido adiposo tiene lugar la forma más rápida de deposición de la grasa en forma de triglicéridos por la alta actividad de la LPL (90%) (Wood *et al*., 2008).

La cantidad de triglicéridos del tejido adiposo va a depender del estado energético del animal, de modo que la síntesis y la degradación obedecen fielmente a las necesidades fisiológicas. Su síntesis va a ser máxima cuando abundan los carbohidratos y la carga energética, y cuando escasean los ácidos grasos. De esta forma cuando el suministro de energía es suficiente para cubrir las necesidades de crecimiento su síntesis va a depender de la cantidad de carbohidratos (Chwalibog *et al.*, 1992). Cuando las necesidades están cubiertas prácticamente toda la grasa del alimento parece retenerse en el cuerpo del animal con pocas modificaciones, por ello existe una estrecha relación entre el tipo de grasa ingerida y la depositada (Miller *et al*., 1990; Larick *et al*., 1992; Hudgins *et al*., 2000).

1.5.2. SÍNTESIS ENDÓGENA DE AG O LIPOGÉNESIS *DE NOVO*

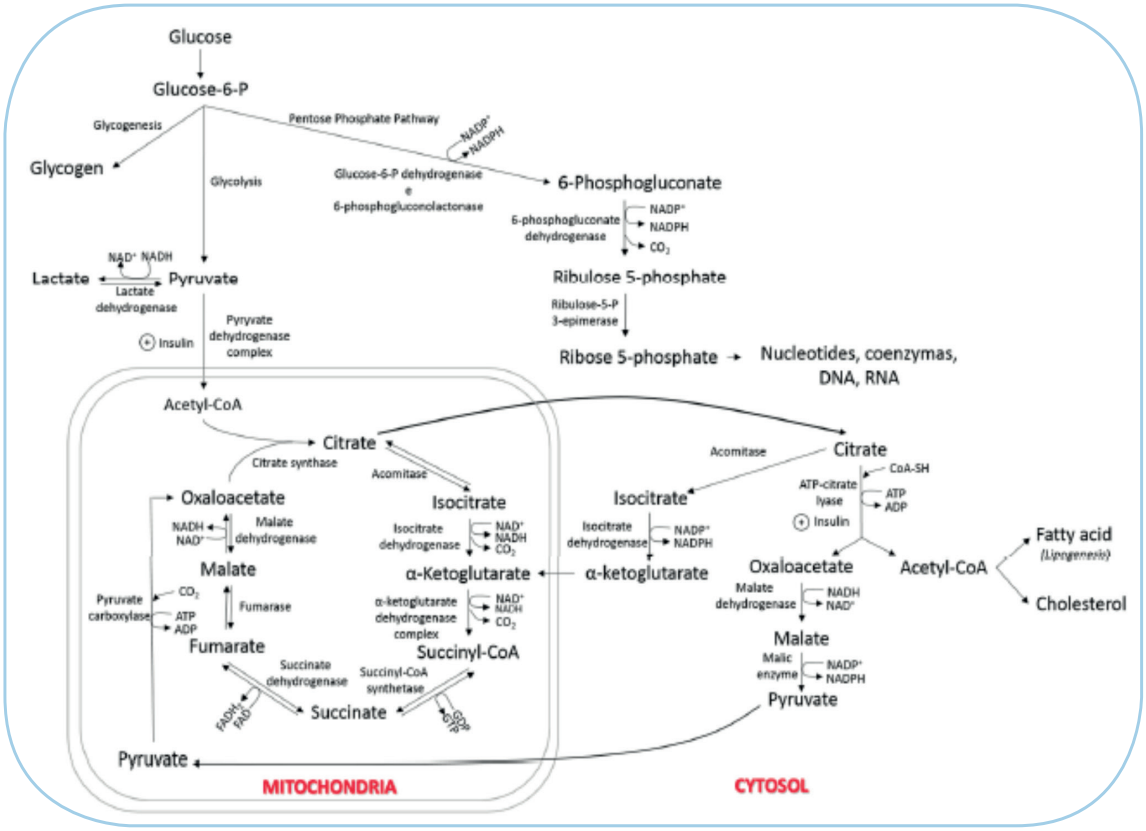
En el cerdo, a diferencia de otras especies, el órgano principal en la síntesis de AG es el tejido adiposo. Tradicionalmente, la composición de las dietas destinadas a los cerdos ha presentado poca cantidad de grasa, por lo que la síntesis *de novo* a partir de carbohidratos ha sido la principal fuente de grasa corporal (O'Hea y

Leveille, 1969). El principal componente de los piensos destinados al ganado porcino son los cereales, ricos en almidón, así la glucosa procedente del almidón es el principal precursor de la síntesis grasa en el cerdo (Mersmann, 1986).

La lipogenésis *de novo* tiene como misión convertir los carbohidratos en lípidos para su almacenamiento, ya que los lípidos tienen más energía que los carbohidratos y son una forma más eficiente de reserva. Los AG y sus derivados son también moléculas señalizadoras que afectan a muchos procesos fisiológicos fundamentales. De este modo la lipogénesis *de novo* puede generar especies lipídicas con bioactividades diferentes de los lípidos procedentes de la dieta. Por tanto, existe un creciente interés por estudiar el papel fisiológico de la lipogénesis *de novo* en la biología normal de los animales y en estados patológicos asociados a la obesidad.

La síntesis *de novo* de los AG ocurre en el citoplasma celular de los adipocitos, para ello se necesita una fuente de carbono (acetil-CoA), una fuente de hidrógeno (NADPH) y una fuente de energía (ATP). El acetil-CoA formado a partir de acetato y coenzima A se transforma en malonil-CoA que es la molécula encargada de ceder dos átomos de carbono que formaran parte del esqueleto carbonado de los AG (Figura 9).

Figura 9: Resumen del metabolismo lipídico (Ladeira et al., 2016).



El acetil CoA es suministrado principalmente por la degradación de la glucosa procedente de la dieta, el NADPH por el ciclo de las pentosas fosfato y el ATP se genera en su mayoría en la cadena respiratoria acoplada a la fosforilación oxidativa mitocondrial.

Los dos sistemas enzimáticos implicados en la síntesis de los AG son: acetil-CoA carboxilasa, que cataliza el paso de acetil-CoA a malonil- CoA y la sintasa de AG que une las moléculas de malonil-CoA para formar el palmitato. El palmitato sintetizado *de novo*, así como los AG de la dieta pueden ser modificados por las elongasas y las desaturasas ubicadas en el retículo endoplásmico para producir otros AG. Estas enzimas están reguladas de manera coordinada con los enzimas de la lipogénesis, de manera que, dependiendo de la batería de enzimas en un tejido específico, el perfil de AG sintetizados *de novo* puede variar y producirse distintos AG con propiedades biológicas diferentes.

Existen dos vías para producir acetil-CoA y los animales utilizaran una vía u otra dependiendo de si son monogástricos, como es el cerdo, o rumiantes debido a la disponibilidad citoplasmática de sus precursores. En el cerdo predomina la vía glicolítica explicada anteriormente y las enzimas participantes son: NADP-malato deshidrogenasa y el enzima málico (Strable and Ntambi., 2010; Ladeira *et al*., 2016).

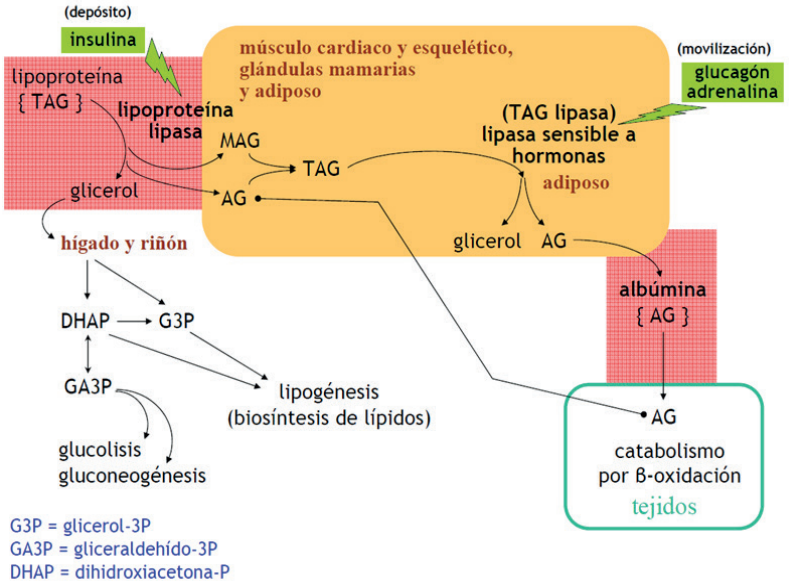
En el hígado y en el tejido adiposo se producirá fundamentalmente ácido palmítico, que es el principal precursor de los AG de cadena larga en las células animales.

1.5.2.1. Regulación del metabolismo lipídico.

Después de la ingesta de alimentos aumentan los niveles de glucosa e insulina en plasma y se inhibe la lipolisis, almacenándose los lípidos procedentes de la lipogénesis y de la dieta. Sin embargo el ayuno promueve la movilización de los AG libres al disminuir el nivel de insulina en plasma y aumentar los de adrenalina y noradrenalina.

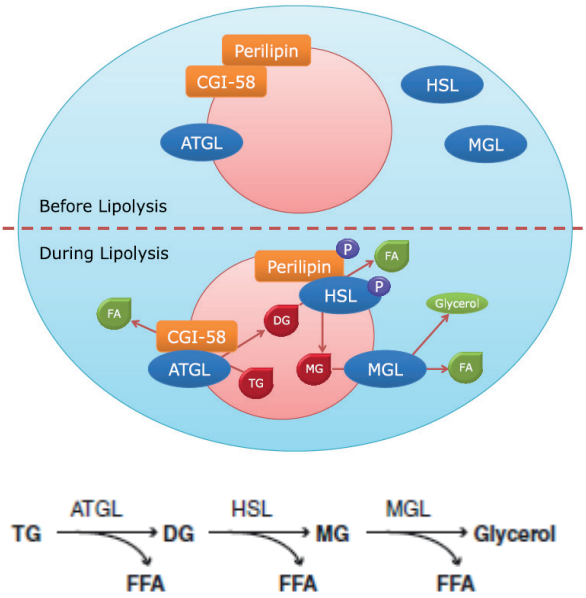
En la lipolisis, los AG libres son liberados a partir de los triacilgliceroles desde el adipocito para la β-oxidación. Estos AG libres, unidos a la albumina, son transportados del tejido adiposo a otros tejidos como el músculo y el hígado para su utilización. Alternativamente pueden ser re-esterificados y entrar de nuevo en la lipolisis, estando este proceso regulado por las hormonas lipolíticas (ATGL, HSL y MGLL) (Figura 10ª y 10ᵇ). La tasa de absorción de los AG libres por estos tejidos es proporcional a su concentración en sangre (Luglio *et al*., 2015).

Figura 10a. Lipolisis y catabolismo de los triacilgliceroles. Fuente DocPlayer



En todos los tejidos la tasa de β-oxidación es regulada por la disponibilidad de la CoA, la cual es regenerada siguiendo la utilización de acetil-CoA por la cetogenesis en hígado y por la enzima citrato sintetasa en músculo. En hígado la β-oxidación está regulada por el control de la absorción de los AG en la mitocondria por el sistema de la Carnitina. En el músculo se regula por la disponibilidad de CoA, NAD^+ y FAD los cuales son generados en sus formas reducidas cuando el ATP es producido en la fosforilación oxidativa en el músculo en ejercicio.

Figura 10b: Proceso de lipolisis en el adipocito (Luglio *et al*., 2015; Nielsen *et al*., 2014)



Como ocurre con otros metabolitos, la síntesis y oxidación de las grasas se producen en diferentes compartimentos celulares y se controlan directamente por el nivel y la actividad de distintas enzimas. La disponibilidad enzimática puede ser regulada a nivel de transcripción (síntesis de RNA mensajero, procesamiento y estabilidad), la traducción (síntesis de proteína a partir de RNA mensajero) o a nivel de post-traducción.

Además, la transcripción depende de la actividad de varios factores de transcripción, algunos de ellos implicados en la síntesis y oxidación de los ácidos grasos y fundamentalmente en la adipogenesis como los PPAR, LXR, o SREBP. Estos factores de transcripción son esenciales para la regulación de la expresión (síntesis) de varios enzimas clave implicados en las vías que controlan el metabolismo lipídico (Jump, 2002).

Por tanto la deposición grasa en los tejidos es un proceso que depende del balance entre los procesos de lipogénesis y lipolisis en el organismo (Pena *et al.,* 2014).

1.6. EMPLEO DE LA DIETA PARA MODULAR LA DEPOSICIÓN GRASA Y EL METABOLISMO LIPÍDICO EN EL CERDO.

Aunque existe una clara contribución de los factores individuales (edad, sexo y genotipo) en la composición de AG de los tejidos, los factores ambientales como la alimentación y el sistema de producción son los que determinan en última instancia la acumulación y síntesis de grasa. En los últimos años se ha explorado qué estrategias nutricionales podemos utilizar para modificar el contenido y la composición de la grasa intramuscular y corporal, especialmente en cerdo. Así podemos distinguir entre modificaciones del nivel de ingestión (*ad libitum* o restricción), del nivel energético y de la composición de la alimentación (contenido de macronutrientes como grasa, proteína e hidratos de carbono y sus proporciones relativas, pero también de micronutrientes como las vitaminas) encaminadas a modular la deposición grasa.

En cuanto al nivel de ingestión, es uno de los factores que más influye en el grado de engrasamiento corporal del cerdo. La deposición grasa depende del balance entre la energía ingerida y los gastos del metabolismo, así la energía que sobra después del mantenimiento y el crecimiento magro se transforma en grasa. Las razas seleccionadas para aumentar el rendimiento cárnico también lo son de forma indirecta para una disminución de la capacidad de ingestión. En el caso de los animales de raza ibérica al ser una raza apenas seleccionada presenta menor crecimiento magro y mayor apetito, lo cual afecta a su elevado grado de engrasamiento (Nieto *et al*., 2002; Barea *et al*., 2007 y Garcia-Valverde *et al*., 2008).

En consecuencia, la restricción en el nivel de ingestión en cerdos ibéricos podría disminuir el nivel de engrasamiento (Serrano *et al*., 2009).

Los carbohidratos constituyen el principal sustrato para la síntesis de grasa en el cerdo. Las dietas ricas en carbohidratos estimulan la actividad enzimática de síntesis de lípidos (Kouba y Mourot, 1998). Tanto *in vitro* como *in vivo* la glucosa estimula la transcripción de estas enzimas en el tejido adiposo y el hígado, aunque el efecto se debe en realidad a su metabolito inmediato la glucosa-6 fosfato (Girard *et al*., 1997). A pesar de no tener efecto directo, la insulina puede ejercer un efecto estimulador de este paso. Los piensos ricos en carbohidratos aumentan la glicemia, desencadenando una respuesta insulinémica que estimula la síntesis de enzimas participantes en la lipogénesis como la acetil-CoA carboxilasa, el enzima málico y la glucosa-6-fosfato deshidrogenasa (Towle *et al*., 1997; Wood *et al*., 2004; Teye *et al*., 2006). El contenido de grasa de la ración también juega un papel importante en la grasa depositada. La presencia de altas concentraciones de grasa en la ración provoca una disminución de las síntesis endógena (Hudgins *et al*., 2000), por lo que el origen de los AG de los tejidos del animal será principalmente el aportado con la dieta (Wood, 1984). Esto es debido a la falta de sustrato (carbohidratos) y también por una regulación metabólica pues ciertos AG provocan la inhibición de las enzimas encargadas de la lipogénesis (Duran-Montgé *et al*., 2007).

En cuanto al nivel energético algunos estudios han demostrado que la composición de AG no se ve afectada por el nivel energético de la dieta (Alonso *et al.,* 2012; Suarez-Belloch *et al.,* 2013), pero si por la fuente de grasa (Weber *et al.,* 2006; Alonso *et al.*, 2012) que determina la concentración de AGMI y AGPI, siendo la concentración de AGS más estable entre distintas fuentes de grasa, debido a que en su concentración influye también la cantidad sintetizada por síntesis endógena y no solo la que reciben de la dieta.

Otra estrategia nutricional que se ha estudiado por su potencial para modular el contenido en grasa ha sido la restricción de la vitamina A, ya que su metabolito activo, el ácido retinoico, influye en los procesos de adipogénesis y lipogénesis (Bonet *et al.,* 2003). Los estudios en cerdo ibérico han mostrado que la restricción de vitamina A en edades tempranas produce una mejor infiltración de grasa en el músculo y un aumento de los AGMI en detrimento de los AGS. Además, se ha comprobado que dichos efectos están relacionados con cambios en la expresión de genes involucrados en la adipogenesis, el metabolismo lipídico y el metabolismo y señalización del retinol (Ayuso *et al.,* 2015a; Ayuso *et al.,* 2015c).

El contenido de proteína de la dieta y más específicamente la relación proteína/energía puede usarse para modificar el grado de engrasamiento de la canal. Cuando la cantidad de proteína en la dieta está por debajo de las necesidades, el cerdo no expresará su potencial de crecimiento magro y el exceso de energía se depositará en forma de grasa (Teye *et al.,* 2006; Rodríguez-Sánchez *et al*., 2011)

Durante el período de engorde del cerdo ibérico en la dehesa, la bellota y la hierba son su principal fuente de alimento. La bellota, pese a ser una excelente fuente de energía, presenta un bajo nivel de proteína y de escasa calidad, siendo la lisina el principal aminoácido limitante (Nieto *et al.,* 2002). De esta forma, la modificación del nivel de lisina en la dieta puede utilizarse como estrategia para modular el contenido graso, ya que reduciendo su nivel y manteniendo constante el nivel de proteína se puede conseguir un incremento en la GIM tanto en la raza ibérica como en otras razas convencionales (Rivera-Ferre *et al.,* 2005; Rivera-Ferre *et al.,* 2006; Palma-Granados *et al.,* 2019).

En el cerdo ibérico cebado con bellotas en la montanera la síntesis endógena parece no muy marcada pues el contenido en AGS (principalmente palmítico y esteárico) en los tejidos no es elevado si no que permanece más o menos constante. Esto puede ser debido a que estos animales utilizan principalmente los carbohidratos del alimento para cubrir las necesidades de mantenimiento, desplazamiento, termorregulación y síntesis proteica y no para sintetizar grasa, siendo los AG de la bellota la fuente principal de grasa animal, como se refleja en el perfil lipídico de estos animales (Rodríguez-Sánchez *et al.*, 2010).

Actualmente en la producción de cerdo ibérico el empleo de dietas enriquecidas en AG especialmente en ácido oleico es una práctica que permite mimetizar el engorde de estos animales en el sistema tradicional de montanera basado en la ingesta de bellotas y pasto. Esto conduciría a una modificación de la composición de AG de los tejidos debido a la acumulación de los componentes de la dieta pero también estos componentes de la dieta contienen elementos bioactivos que podrían modular la síntesis endógena y la actividad enzimática.

Los factores genéticos y nutricionales pueden tener a su vez efectos de interacción sobre el metabolismo tisular ya que diferentes genotipos podrían responder de forma distinta ante los cambios de la composición de los alimentos (Wood *et al*., 2004; Olivares *et al*., 2009; Godinho *et al*., 2018). El conocimiento de los genes y rutas metabólicas involucrados en estos procesos

nos permitiría profundizar en el conocimiento del metabolismo característico del cerdo ibérico.

1.7. METODOLOGÍAS DE ANÁLISIS MOLECULAR APLICADAS A LA MEJORA GENÉTICA ANIMAL

Hay una gran variedad de herramientas moleculares y métodos estadísticos que se están empleando con el fin de conocer la base genética de la regulación de caracteres complejos como el crecimiento y la deposición grasa y para poder profundizar en el conocimiento de la función del genoma a gran escala.

Durante los años 90 comenzaron a realizarse numerosos estudios centrados en las variaciones de la secuencia del ADN especialmente con marcadores de tipo microsatélite, con el fin de construir mapas genéticos y detectar regiones del genoma o "quantitative trait loci" QTL con influencia sobre determinados caracteres de interés económico (Ollivier *et al*., 2009). Estos estudios han permitido identificar abundantes QTL y genes candidato posicionales a lo largo del genoma porcino (www.animalgenome.org/cgi-bin/QTLdb/SS/index) (Hu *et al*., 2016).

En el año 2003 se estableció el consorcio para la secuenciación del genoma porcino (Swine Genome Sequencing Consortium, SGSC) con el objetivo de llevar a cabo su secuenciación y caracterización. El genoma porcino está compuesto por 18 pares de autosomas y dos cromosomas sexuales (cromosomas X e Y) y tiene un tamaño total estimado en 2,8 billones de pares de bases y que contiene 22.452 genes codificantes de proteínas (Gilbert, 2019). Es muy parecido al genoma de otros mamíferos incluido el humano, aunque la variación interindividual en el genoma del cerdo, incluso en las razas comerciales, es más del doble que en los seres humanos. En primer lugar se llevó a cabo la secuenciación de una hembra de la raza duroc usando cromosomas artificiales de bacterias (BACs) obteniendo una cobertura de 4x (Archibald *et al*., 2010). Desde entonces, se han puesto a disposición de la comunidad investigadora diversas versiones del genoma porcino que se han revisado y mejorado hasta la actual versión Sscrofa 11.1 (SGSC, 2017).

La disponibilidad del genoma porcino ha proporcionado cientos de miles de nuevos marcadores genéticos permitiendo un gran avance en la detección de regiones genómicas asociadas a caracteres de interés. Las limitaciones que tenían los marcadores microsatélite se han superado con el uso de marcadores tipo SNP (single nucleotide polymorphism) y la aparición de los chips de genotipado masi-

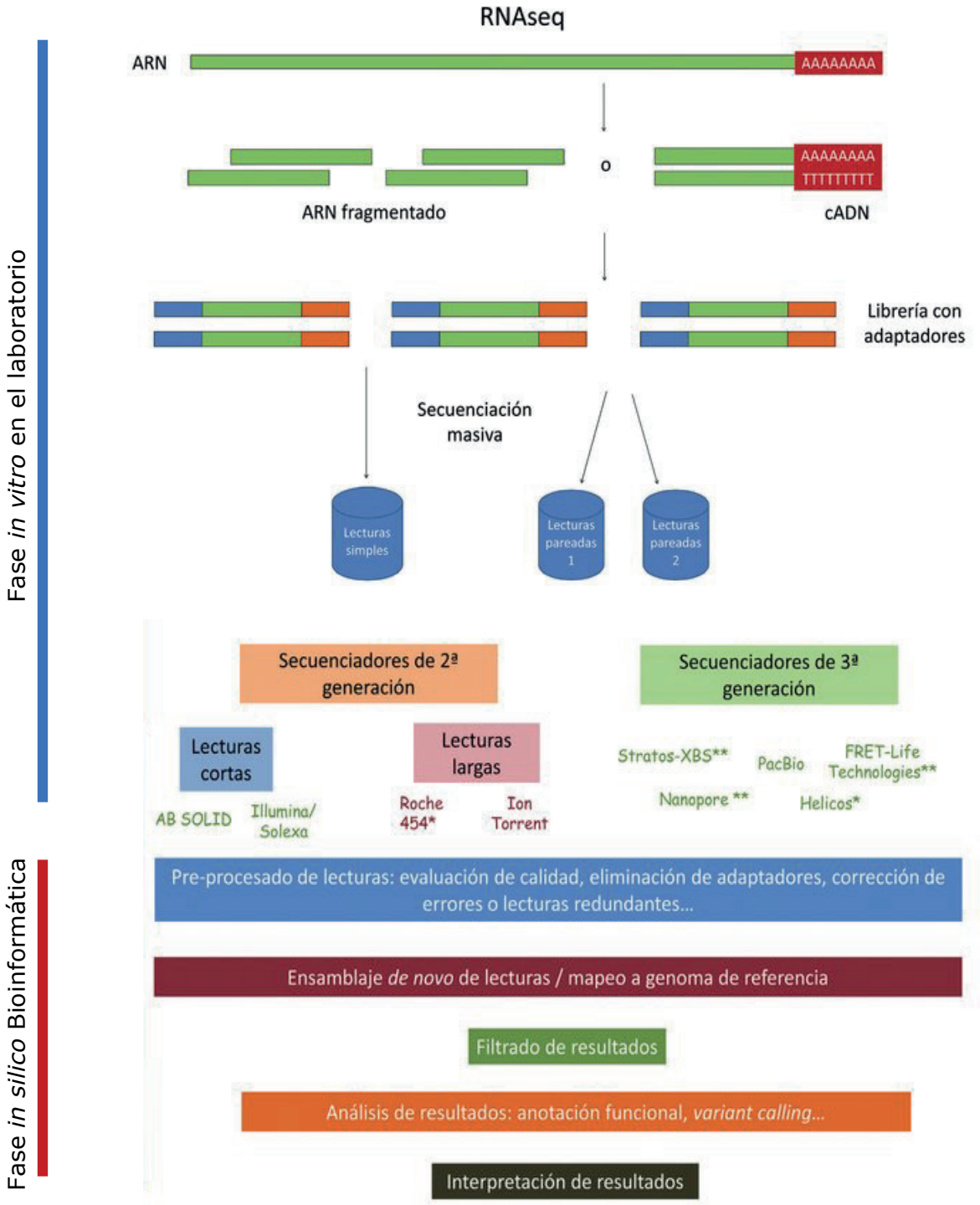
vo. Los SNPs son marcadores bialélicos menos informativos que los microsatélites, pero su abundancia y automatización en el genotipado los han convertido en los marcadores más eficientes y más utilizados. Con la creación de los chips de genotipado masivo de SNPs para todas las especies de interés ganadero, incluido el cerdo (Ramos *et al*., 2009), se consigue no solo refinar la posición de los QTLs ya identificados en estudios GWAS, sino identificar nuevos en regiones que no estaban bien cubiertas, obteniendo unos intervalos de confianza más reducidos que nos facilitan la búsqueda de genes y mutaciones causales.

La secuenciación del genoma porcino y el espectacular desarrollo de los métodos de análisis masivo del genoma han permitido a su vez el avance en el desarrollo y aplicación de las tecnologías de análisis del transcriptoma. En primer lugar se desarrollaron los microarrays, que permitían analizar la expresión de miles de genes conocidos. En concreto para la especie porcina se desarrollaron dos chips comerciales, el chip de la plataforma Affymetrix Porcine Gene ChipTM, que contiene 23,937 conjuntos de sondas correspondientes a 23,256 tránscritos de 20,201 genes conocidos y el Agilent Porcine Gene Expression Microarray que contiene 43,803 sondas. Sin embargo, en los últimos años la rápida evolución de las tecnologías de secuenciación masiva han revolucionado las técnicas de análisis global de la expresión génica. La secuenciación masiva del transcriptoma realizada mediante la técnica conocida como RNA-seq permite analizar la gran complejidad del transcriptoma generando una visión global y permitiendo un análisis mucho más exhaustivo (Chen *et al*., 2011).

Esta estrategia presenta varias ventajas frente al uso de microarrays. En primer lugar, tiene mayor sensibilidad y rango dinámico y menor variación técnica y ruido, además requiere menor cantidad de ARN de partida (Oshlack *et al*., 2010; Chen *et al*., 2011). En segundo lugar, mediante RNA-seq es posible capturar prácticamente todos los tránscritos expresados, mientras que los análisis basados en microarrays dependen de información a priori y no son capaces de detectar nuevos genes o tránscritos. Permite también la cuantificación de la expresión de cada tránscrito, de ARNs no codificantes y de mutaciones post transcripcionales (Wang *et al*., 2009). Por último, nos permite investigar no sólo cambios en la expresión génica, sino también cambios estructurales en el ARN mensajero como SNPs y otras variantes estructurales, lo que permite a su vez determinar la expresión génica de forma alelo-específica (Qian *et al*., 2014).

El procedimiento habitual en este tipo de análisis (Figura 11) consiste en la fragmentación del ARN y secuenciación de fragmentos cortos mediante alguna de las tecnologías disponibles en el mercado. En la actualidad existen secuenciadores de 2ª generación como los de Illumina/Solexa, Roche o Ion Torrent, que generan secuencias de entre 35 y 500 pb (Martin y Wang, 2011) o secuen-

Figura 11: Esquema general de secuenciación del transcriptoma.



ciadores de 3ª generación como los de PacBio o Oxford Nanopore, con los que se consiguen lecturas muy largas y además tienen la ventaja añadida de que no necesitan pre-amplificación por PCR (Kukurba & Montgomery, 2015).

Posteriormente estas lecturas son mapeadas frente al genoma de referencia, en el caso de estar disponible, o alineadas *de novo* si no existe referencia. En un tercer paso, las lecturas son ensambladas en fragmentos de mayor tamaño dentro de los genes o tránscritos. El análisis del nivel de expresión de cada tránscrito puede realizarse debido a que el número de lecturas obtenidas es proporcional al nivel de expresión. Así, una vez normalizados los datos es posible obtener los niveles de expresión de cada tránscrito y realizar un análisis de expresión diferencial (Oshlack *et al*., 2010).

El objetivo de un estudio de expresión diferencial consiste en identificar los genes cuya expresión ha cambiado significativamente entre dos condiciones diferentes. Es posible identificar no solo genes diferencialmente expresados (DE) sino también isoformas DE, diferente uso de promotores y diferentes sitios de inicio de la transcripción (Trapnell *et al*., 2012).

A pesar de las grandes ventajas de la tecnología RNA-Seq, también presenta algunas limitaciones, desde fallos en el procesamiento y secuenciación de las muestras hasta la complejidad del análisis bioinformático de los datos. La mayoría de los sesgos en la construcción de las librerías y en la secuenciación quedan solventados con el uso de lecturas pareadas. Sin embargo, es necesario seguir implementando los métodos de análisis de los datos obtenidos, además de homogeneizar el protocolo de análisis, lo que resulta complicado teniendo en cuenta la cantidad de programas informáticos y estrategias de análisis disponibles y en continuo desarrollo. Por otro lado, la cantidad de información que generan este tipo de experimentos (del orden de 5 GB por archivo), encarece y dificulta el almacenamiento y procesado (Mantione *et al*., 2014; Chen *et al*., 2011).

Los estudios de expresión diferencial también se han realizado tradicionalmente por medio de PCR cuantitativa a tiempo real con una aproximación de genes candidato. Además la tecnología de la PCR cuantitativa a tiempo real se utiliza habitualmente para la validación técnica de los estudios de análisis del transcriptoma ya sea con la técnica de los microarrays o de RNA-seq.

Estas dos metodologías de análisis del transcriptoma (microarrays y RNA-Seq) se han aplicado de forma extensa en la especie porcina con el fin de entender los aspectos genéticos asociados a caracteres de interés. Numerosos estudios han

investigado las bases genéticas relacionadas con el desarrollo muscular (Cagnazzo *et al*., 2006; Kim *et al*., 2010; D'Andrea *et al*., 2011; Zhao *et al*., 2011; Damon *et al*., 2012; Guo *et al*., 2014; Óvilo *et al*., 2014b), que históricamente han despertado un gran interés en los científicos debido a la importancia económica que supone el desarrollo muscular en la producción porcina y en la de otras especies ganaderas, así como la resistencia a enfermedades y desarrollo del sistema inmune (Bao *et al*., 2012; Mach *et al.*, 2013; Xing *et al*., 2014). También se ha estudiado el transcriptoma en relación a caracteres reproductivos (Samborski *et al*., 2013) y a factores relacionados con la calidad de la carne, como pueden ser la composición de ácidos grasos (Puig-Oliveras *et al*., 2014) o el engrasamiento (Pérez-Montarelo *et al*., 2014; Xing *et al*., 2015). La mayoría de estos estudios se ha realizado en razas comerciales (Sodhi *et al*., 2014) y en una gran variedad de tejidos. Los trabajos orientados al descubrimiento de los genes implicados en el crecimiento o desarrollo muscular se centraron preferentemente en el transcriptoma muscular, aunque también se han estudiado otros tejidos, como por ejemplo la grasa dorsal (Corominas *et al*., 2013a) o tejidos con función endocrina como la glándula tiroides, las gónadas (Pérez-Enciso *et al*., 2009) o el hipotálamo (Pérez-Enciso *et al*., 2009; Pérez-Montarelo *et al*., 2014).

La aplicación de estas técnicas en razas autóctonas como la ibérica es más reducida y se basa sobre todo en la comparación del transcriptoma de razas locales con razas comerciales, habitualmente muy divergentes de las primeras. En el caso del cerdo ibérico, los trabajos realizados en este ámbito son escasos (Tabla 2).

Un buen número de estudios en los que se ha analizado el transcriptoma del cerdo ibérico puro o cruzado, han estado encaminados a encontrar genes diferencialmente expresados (DE) en animales con fenotipos divergentes para ciertos caracteres relacionados con parámetros productivos o de calidad de carne, como el crecimiento o la cantidad y la composición de la GIM o la grasa subcutánea. Para ello, se han empleado cruces experimentales de cerdo ibérico con razas muy diferentes desde un punto de vista fenotípico y genético, lo que facilita la obtención de animales con una gran variabilidad. Estos estudios (Ramayo-Caldas *et al*., 2012; Corominas *et al*., 2013a; Perez-Montarelo *et al*., 2014; Puig-Oliveras *et al*., 2014) permitieron identificar genes DE y rutas metabólicas enriquecidas entre cerdos de fenotipos extremos relacionados con el crecimiento, el engrasamiento o la composición de la grasa. La información

generada sobre los mecanismos genéticos involucrados en estas diferencias fenotípicas es muy útil para diseñar futuros estudios que permitan identificar mutaciones causales en alguno de los genes candidato propuestos previamente, lo que repercutiría económicamente en el sector porcino. Debe tenerse en cuenta, no obstante, que los estudios basados en la comparación de razas divergentes o en animales obtenidos por cruzamiento entre ellas, identifican preferentemente diferencias genéticas entre estas razas, pero no son óptimos para la identificación de la variación intrapoblacional. Por otra parte, los resultados de estos trabajos también pueden ser relevantes desde el punto de vista de la salud humana, puesto que muchos de estos mecanismos relacionados con el crecimiento y el metabolismo y acumulación de la grasa, pueden ser comunes entre ambas especies, lo que contribuiría a la investigación de enfermedades como la obesidad, el síndrome metabólico o la diabetes tipo 2, (Torres-Rovira *et al*., 2012; Barbero *et al*., 2014; Óvilo *et al*., 2014c).

Más recientemente, Óvilo y colaboradores (2014) realizaron una comparación del transcriptoma del músculo *Longissimus dorsi* LD del cerdo ibérico con un tipo genético mucho más cercano, el cruce comercial habitual de ibérico con duroc (50%), mediante microarrays de expresión. Estos dos tipos genéticos son de gran importancia para el sector del porcino ibérico, puesto que son los más importantes entre los aceptados por el Real Decreto 4/2014 modificado por el Real Decreto 255/2016, para la obtención y elaboración de productos etiquetados como "ibérico". Pese a la cercanía de los genotipos comparados y a la temprana edad a la que se realizó el estudio (28 días), se encontraron diferencias marcadas, tanto a nivel fenotípico (los animales puros presentaron mayor GIM que los cruzados) como a nivel de expresión, detectando 250 genes DE que además se relacionaron con procesos tan interesantes desde el punto de vista del desarrollo muscular y la acumulación de grasa, como el desarrollo de la matriz extracelular, la proteólisis o el metabolismo lipídico. En este trabajo se identificaron además potenciales genes reguladores responsables de las diferencias en expresión génica y por lo tanto en características fenotípicas entre ambos tipos genéticos. En 2016 Ayuso y colaboradores emplearon la técnica RNAseq para investigar los efectos del tipo genético (ibérico puro *vs* duroc x ibérico) y del músculo (*Longissimus dorsi vs Biceps femoris*) sobre la expresión génica y la regulación transcripcional en dos etapas tempranas del desarrollo:

al nacimiento y en periodo de crecimiento. Este estudio revela los posibles mecanismos moleculares responsables de las diferencias fenotípicas observadas entre puros y cruzados y destaca los genes candidato implicados en estos mecanismos. En 2017, Cardoso y colaboradores identificaron genes diferencialmente expresados y sus isoformas en músculo esquelético de cerdos duroc con perfiles lipídicos extremos, estudiando su implicación en la deposición grasa y en la calidad de la carne, además de determinar la contribución relativa de los ARN codificantes y no codificantes en la expresión diferencial.

Tabla 2: Estudios del transcriptoma realizados en animales de raza ibérica y otros genotipos relacionados.

Tecnología	Objetivo	Tejido	Raza/s	Referencia
Microarray	Comparación animales extremos para composición de AG	Músculo	IB x LD	(Pena et al., 2013)
RNA Seq	Comparación entre razas	Testículo	IB y LW	(Esteve-Codina et al., 2011)
Microarray	Comparación animales extremos para prolificidad	Ovario	IB x MS	(Fernandez-Rodriguez et al., 2011)
RNA Seq	Comparación animales extremos para composición de AG	Músculo	IB x LD	(Puig-Oliveras et al., 2014)
RNA Seq	Comparación animales extremos para crecimiento y engrasamiento	Hipotálamo	IB x LD	(Perez-Montarelo et al., 2014)
RNA Seq	Comparación animales extremos para composición de AG en GIM	Grasa	IB x LD	(Corominas et al., 2013)
Microarray	Efecto de la fuente de energía de la dieta	Grasa	IB	(Óvilo et al., 2014a)
RNA Seq	Comparación animales extremos para composición de AG en GIM	Hígado	IB x LD	(Ramayo-Caldas et al., 2012)
Microarray	Comparación entre tipos genéticos	Músculo	IB, IB x DU	(Óvilo et al., 2014b)
RNA Seq	Comparación entre tipos genéticos	Músculos (LD y BF)	IB, IB x DU	(Ayuso et al., 2015b y Ayuso et al, 2016)
RNA Seq	Comparación diferentes perfiles lipídicos	Músculo	Híbridos	(Cardoso et al, 2017a)
RNA Seq	Efecto restricción % PB extremos GIM	Hígado y L. dorsi	Ib x DU	(Muñoz et al., 2018)

IB: ibérico, LW: Large White, MS: Meishan, AG: Ácidos grasos, GIM: Grasa intramuscular, DU: duroc, LD: Longissimus dorsi y BF: Biceps femoris.

Pese a existir varios estudios comparando el transcriptoma de cerdos ibéricos en pureza con animales cruzados con duroc no existen estudios que comparen ambas razas en pureza y en idénticas condiciones de manejo lo que nos proporcionaría una valiosísima información acerca de las particularidades fisiogenéticas y metabólicas de estas dos razas.

1.8. GENÓMICA NUTRICIONAL. NUTRIGENÓMICA Y NUTRIGENÉTICA

En la actualidad el enorme desarrollo de las técnicas de genética molecular que permiten el estudio del genoma a gran escala, la disminución en los costes, así como una mayor accesibilidad han contribuido al desarrollo de nuevas disciplinas que se englobarían dentro del término genérico de genómica nutricional. Esta disciplina estudia las interacciones de los alimentos y sus componentes con el genoma a nivel molecular, celular, y sistémico. La genómica nutricional se divide en dos campos de investigación claramente diferenciados: la nutrigenómica y la nutrigenética, nuevas disciplinas que estudian los efectos de los alimentos a nivel genético.

La Nutrigenómica propiamente dicha, estudia como los nutrientes de la dieta influyen en la homeostasis celular, alterando la expresión génica y la producción de proteínas y/o metabolitos. En este campo se han realizado bastantes estudios en humano y ratón planteándose como principales objetivos el estudio de la base molecular de enfermedades como la obesidad, las enfermedades cardiovasculares y el cáncer (López *et al.*, 2003; Ordovás & Corella 2004). En animales domésticos estos estudios nutrigenómicos son mucho menos abundantes y en general están enfocados a un número reducido de genes relacionados especialmente con un determinado tratamiento, generalmente referido al contenido energético de la dieta, o al contenido de algunos de sus componentes: ácidos grasos poliinsaturados (PUFA) (Hsu & Ding, 2003), proteína (Hamill *et al.*, 2013), L-carnitina (Keller *et al.*, 2011) o vitaminas (Ayuso *et al*., 2015a,c y d).

La Nutrigenética se ocupa de caracterizar cómo las distintas variantes del genoma influyen en la respuesta del organismo a los nutrientes. El objetivo último de esta disciplina aplicada es proporcionar recomendaciones nutricionales teniendo en cuenta la constitución genética de cada individuo, lo que en nutrición humana se llama nutrición personalizada. En animales domésticos esta disciplina está prác-

ticamente inexplorada, aunque existen algunos trabajos cuyo enfoque se puede considerar nutrigenético como el estudio de la relación de las variantes del gen *SCD* con el efecto de la suplementación con vitamina A en cerdos (Henriquez-Rodriguez *et al*., 2017). Los resultados obtenidos en este trabajo proporcionan una evidencia del papel complementario del contenido en provitamina A de la dieta y el genotipo del gen *SCD*, en el sentido que los cerdos de genotipo TT y alimentados con una dieta de bajo contenido en provitamina A muestran un contenido en GIM más alto y más monoinsaturado sin aumentar el contenido de grasa total.

El uso de herramientas multidisciplinares brinda nuevas oportunidades para investigar las interacciones complejas del genoma y la dieta. Durante la última década se ha reconocido que determinados lípidos de la dieta tienen actividades biológicas únicas debido a sus efectos estimuladores/inhibidores sobre la transcripción de genes que codifican para enzimas que intervienen en el metabolismo lipídico (Jump, 2002). Esto supone que es posible modificar el perfil de ácidos grasos de los diferentes depósitos grasos del cerdo ibérico con la dieta, debido no solo a los procesos de deposición directa sino al aprovechamiento de las propiedades bioactivas de los nutrientes (Tejeda *et al*., 2002; Ventanas *et al*., 2007; Óvilo *et al*., 2014a).

Por otro lado también es interesante explorar los mecanismos moleculares que se desencadenan como consecuencia del consumo de alimentos y después de un periodo de ayuno. Algunos estudios muy recientes indican que la ingestión/ayuno de alimentos afecta la expresión de muchos factores de transcripción que son esenciales para coordinar la respuesta metabólica provocada por la disponibilidad de nutrientes (Cardoso *et al*., 2017b). Estos estudios sugieren que la respuesta metabólica a la ingesta de alimento, con activación de múltiples factores de transcripción podría estar regulada por los genes que integran el reloj circadiano y que, a su vez, la variación genética de los mismos podría tener efectos importantes sobre el depósito y la composición de los depósitos grasos. (Ribas-Latre & Eckel-Mahan, 2016). A su vez, las oscilaciones en la expresión de los genes que constituyen dichos relojes tienen un efecto amplio y profundo sobre el metabolismo.

El estudio de los genes expresados diferencialmente en animales sometidos a distintos tipos de dieta permite conocer la base molecular de las diferencias fenotípicas y metabólicas inducidas por la dieta, identificando los genes y rutas metabólicas directamente implicadas en la regulación de la composición de los tejidos y por tanto en su calidad.

1.8.1. ESTUDIOS NUTRIGENÓMICOS EN CERDO IBÉRICO

La producción de cerdo ibérico es un claro ejemplo de sistema productivo orientado a la obtención de productos cárnicos de alta calidad. Además del genoma, los aspectos ambientales y de manejo (especialmente los nutricionales) tienen una influencia decisiva en la composición de los tejidos de esta raza y son determinantes en la calidad de sus productos.

Por ello, paralelamente a los estudios propiamente genéticos se han realizado esfuerzos para explorar las posibilidades que ofrece controlar la cantidad y composición de los lípidos que se incluyen en los piensos que se emplean para la mayoritaria producción de cerdos de cebo (López Bote *et al*., 1999, 2000). Además, aparte de los efectos directos de la genética y la nutrición, es necesario tener en cuenta la interacción entre ambos.

Los principales sistemas de producción de cerdos ibéricos se diferencian por la duración del periodo productivo, el nivel de restricción de alimento anterior al periodo de engorde y por la composición de la dieta en el periodo final de cebo. La montanera supone la ingesta de bellotas y pasto durante este último, que conduce a un cambio en el perfil lipídico del músculo y la grasa, con un incremento de AGMI, principalmente ácido oleico, triglicéridos y fosfolípidos; y una reducción de los ácidos palmítico, esteárico y linoleico, en relación a los animales alimentados con pienso concentrado. Este característico perfil de ácidos grasos tiene una gran repercusión en la calidad de los productos curados, ya que los lípidos insaturados y el tocoferol influyen en la oxidación lipídica responsable de la formación de compuestos volátiles que afectan a su aroma y sabor. El empleo de dietas enriquecidas en AG es una posible estrategia para modular la composición de los tejidos y mejorar su calidad organoléptica y tecnológica, como ya se ha comentado anteriormente.

Como consecuencia, desde hace algunos años se ha propuesto la utilización en los sistemas de cebo intensivo de dietas enriquecidas en grasa (hasta el 7-9%), basadas en productos de origen vegetal (girasol, oleínas, etc.) con elevado contenido de ácido oleico (C18:1), intentando imitar la composición de ácidos grasos de la bellota (González *et al.*, 2006).

Por otra parte, la relevancia del control de la composición de la grasa en el cerdo está relacionada con importantes aspectos nutricionales de los alimentos de origen animal (ácidos grasos aterogénicos relacionados con las enfer-

medades cardiovasculares, estabilidad oxidativa de los tejidos, etc.) y con las características sensoriales y tecnológicas de la carne y los productos cárnicos transformados. Aunque es un tema controvertido, entre los AGS que se consideran negativos por tener un efecto desfavorable para la salud, tenemos los de cadena media y larga como ácido laúrico (C12:0), ácido mirístico (C14:0) y en menor medida el ácido palmítico (C16:0). Estos AGS tienen un efecto reducido sobre la consistencia de la grasa. En cambio el ácido esteárico (C18:0) se considera escasamente aterogénico, es decir más saludable, y afecta de forma positiva a la consistencia de la grasa. Algo similar puede indicarse de los AGMI, cuyo interés desde el punto de vista dietético es incuestionable y al mismo tiempo tienen un efecto mucho más moderado sobre la consistencia de la grasa que los AGPI (López Bote *et al*., 2002).

Hasta el momento, los estudios nutrigenómicos en cerdo ibérico han centrado el esfuerzo en explorar las posibilidades que ofrece controlar la cantidad y composición de los lípidos que se incluyen en la alimentación de los cerdos (López Bote *et al*., 1999) para intentar identificar los genes modulados por la dieta y asociados al metabolismo lipídico u otras funciones biológicas relevantes, y que podría tener un interés práctico a la hora de obtener productos con mejor calidad tanto sensorial como nutricional.

La posterior caracterización estructural de estos genes y la identificación de su variación alélica en los cerdos ibéricos presentan implicaciones futuras de gran interés tanto para la mejora genética de la raza como para la formulación de dietas adaptadas al perfil genético de los animales.

El principal antecedente de los trabajos que comprende esta tesis, es un trabajo en cerdo ibérico en el cual se estudiaron los efectos sobre la composición tisular y el metabolismo lipídico en tejidos adiposo, muscular y hepático de animales cebados con dietas isoenergéticas e isoproteicas de distinta composición lipídica (Óvilo *et al*., 2014a). En este ensayo, se comparaban una dieta suplementada con 6% de girasol de alto oleico (HO) y otra basada en carbohidratos como fuente de energía (CH), en animales en crecimiento y a peso final y su efecto a nivel de composición tisular y sobre la expresión génica en diferentes tejidos de cerdos ibéricos. Los resultados de composición mostraron una mayor proporción de grasa saturada en animales cebados con pienso basado en carbohidratos (CH), mientras que los animales cebados con pienso enriquecido con girasol alto

oleico (HO) presentaron más grasa monoinsaturada en todos los tejidos analizados ($P<0.001$) (Tabla 2). Los resultados fueron muy claros en el tejido adiposo, pero menos en tejido muscular y hepático. El efecto de la dieta sobre los AGPI fue pequeño y condicionado al momento del muestreo, al tejido y a la fracción lipídica (lípidos neutros o polares). Estos efectos se establecieron temprano durante el tratamiento y aumentaron solo ligeramente a lo largo del tiempo.

Tabla 3. Composición (% de AG e índices) de la grasa subcutánea

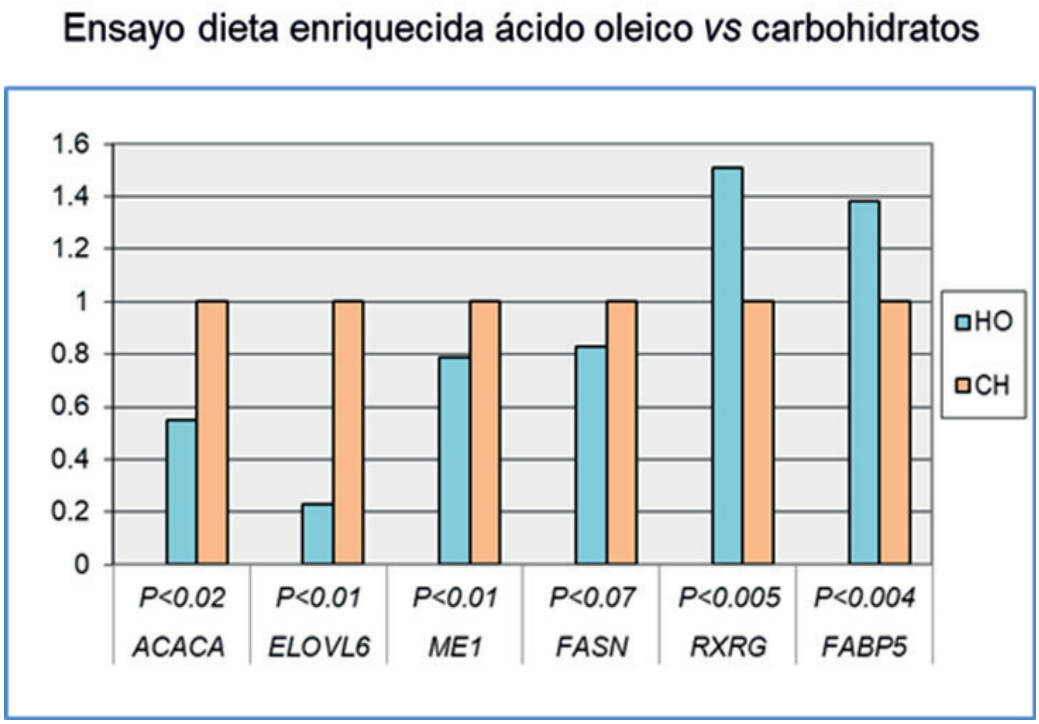
GRASA SUBCUTANEA EXTERNA	ENSAYO CARBOHIDRATOS vs OLEICO		
ACIDOS GRASOS	GRUPO CH	GRUPO HO	
	MEDIA (SEM)	MEDIA (SEM)	*P*-VALUE
C18:1n9	51.2 (0.3)	60.4 (0.3)	<0.001
C18:2	7.0 (0.2)	7.7 (0.2)	0.002
SFA	36.6 (0.4)	26.94 (0.4)	<0.001
MUFA	55.0 (0.3)	63.9 (0.4)	<0.001
PUFA	8.4 (0.2)	9.2 (0.2)	0.01
n-6/n-3	9.9 (0.2)	11.8 (0.2)	<0.001

El efecto de la dieta sobre la expresión génica fue abordado con dos aproximaciones complementarias: analizando el transcriptoma del tejido adiposo con microarrays y estudiando un panel de genes candidato clave relacionados con los procesos de lipogénesis y el metabolismo lipídico en la grasa dorsal, el músculo y el hígado. En el estudio transcriptómico se identificaron 37 genes expresados diferencialmente (DE) relacionados con varias funciones biológicas, incluidos los procesos relacionados con el metabolismo lipídico. Aunque el efecto de la dieta sobre el transcriptoma de la grasa dorsal fue pequeño en cantidad y magnitud, los resultados de la interpretación funcional indicaron que los nutrientes empleados modulaban la expresión de genes relacionados con importantes rutas del metabolismo lipídico, como la concentración y oxidación de ácidos grasos y la síntesis de prostanoides. Los animales del grupo HO presentaron inducción de genes implicados en la protección frente a estrés oxidativo y daño celular, como *UCP3* (uncoupling protein 3), en concordancia con un potencial efecto beneficioso del ácido oleico sobre la salud del animal. Por el contrario la dieta CH estimuló la expresión de mediadores de inflamación como *PTGS2* (prostaglandina endoperóxido sintasa), posiblemente como consecuencia de la mayor acumulación de

grasa saturada en los tejidos de estos animales. El estudio de los genes relacionados con la lipogénesis en tejido adiposo (grasa subcutánea) mostró una mayor expresión de los genes *RXRG* (Receptor X retinoide gamma) y *FABP5* (fatty acid binding protein 5, epidermal) en el grupo HO y los genes *ACACA* (acetil coA carboxilasa), *ELOVL6* (elongasa 6 de ácidos grasos), *ME1* (enzima málico) y *FASN* (sintasa de ácidos grasos) mostraron una mayor expresión en el grupo alimentado con carbohidratos reflejando que la dieta CH estimula la lipogénesis *de novo* de AG en concordancia con los resultados de composición. La PCR cuantitativa confirmó varios genes DE en el tejido adiposo, pero no se encontró ningún gen DE en lomo o hígado. Los resultados en conjunto concuerdan con un ajuste metabólico de los niveles de AG en el tejido adiposo por el efecto sutil de la dieta en la regulación de varias importantes rutas metabólicas de los lípidos, principalmente la oxidación de AG y la síntesis de prostanoides, rutas en las que los genes *LEP, RXRG* y *PTGS2* desempeñan un papel importante (Figura 12).

Se observaron algunas diferencias en la respuesta entre los tejidos, siendo la composición muscular la menos sensible a la dieta y la que presenta la mayor heterogeneidad en la respuesta de las diferentes fracciones lipídicas.

Figura 12: Expresión de genes candidato en la capa interna de la grasa subcutánea.



Los resultados de este ensayo en conjunto indican que la dieta HO produce tejidos animales ricos en ácido oleico y con una menor saturación de la grasa con respecto a la dieta CH. Los resultados indican posibles efectos beneficiosos de una larga suplementación de la dieta con ácido oleico contra el estrés oxidativo y el daño en el ADN. Además los resultados nos permiten resaltar la importancia de la nutrición sobre los distintos procesos fisiológicos y metabólicos con transcendencia a nivel productivo en el cerdo ibérico.

Las peculiares características de la raza ibérica con su sistema tradicional de producción hacen que sea una raza en la que las interacciones genética-nutrición tengan un papel fundamental en el fenotipo. Por tanto la raza ibérica se puede considerar un material animal idóneo para este tipo de ensayos nutrigenómicos en los que se busca explorar en detalle la influencia de factores nutricionales sobre los diferentes procesos fisiogenéticos que afectan a la acumulación de la grasa y al metabolismo de los ácidos grasos en los tejidos y por tanto a la calidad de la carne y de la grasa.

2. JUSTIFICACIÓN Y OBJETIVOS

El cerdo ibérico es una raza que se caracteriza por un crecimiento lento, un escaso desarrollo muscular y una gran capacidad de acumulación de grasa, además de poseer una alta capacidad de adaptación al medio y rusticidad. Al igual que otras razas autóctonas tradicionales, el rendimiento de esta raza es menor al de las razas seleccionadas, lo que suele ir asociado a una menor rentabilidad, suponiendo una amenaza para la sostenibilidad de la producción. Sin embargo, este no es el caso del cerdo ibérico, debido a que cuenta con una diferencia esencial: una carne reconocida en el mercado por su altísima calidad, que se refleja en el alto valor económico de sus productos y que compensa su menor rendimiento productivo. Por ello, de forma tradicional el sector se ha preocupado por mantener e incluso mejorar esta alta calidad de su carne y aumentar su rendimiento productivo. Con el objetivo de mejorar su rendimiento productivo, la raza ibérica se ha cruzado de forma habitual con la raza duroc procurando no menoscabar su calidad, estando la mayoría de la producción del sector del ibérico englobada dentro de los animales de cebo intensivo y cruzados al 50-75% con la raza duroc. Sin embargo, aunque numéricamente su producción sea menor, la mayor relevancia es para la producción de animales 100% ibéricos y de bellota, por su excelente calidad y su gran valor económico.

Los factores que determinan la calidad de su carne y grasa derivan de las características propias de la raza y del sistema de producción en extensivo (López-Bote, 1998). Entre ellos, destaca por su interés la cantidad de grasa en los tejidos y su composición (el contenido en AGMI principalmente ácido oleico) (Ventanas *et al*., 2006). La cantidad de grasa, así como su composición son parámetros muy variables, determinados en gran parte por el genoma del animal. Además del genoma, los aspectos ambientales y de manejo (especialmente los nutricionales) tienen una influencia decisiva en la composición de los tejidos y son determinantes en la calidad de los productos.

Debido a la necesidad de mantener la calidad de la carne tras la incorporación de genética duroc a la raza ibérica, los efectos de este cruzamiento se han estudiado desde un punto de vista fenotípico (Ventanas *et al*., 2007; Serrano *et al*., 2008; Fuentes *et al.*, 2014), así como desde un enfoque más básico encaminado a profundizar en los mecanismos moleculares y genéticos implicados en las diferencias entre genotipos (Pena *et al*., 2013; Óvilo *et al*., 2014; Ayuso *et al*., 2015b; Ayuso *et* al., 2016). Los estudios previos se han centrado principalmente

en la comparación de los dos tipos genéticos productivos (ibéricos puros y cruzados con duroc). Sin embargo, lo particular del metabolismo de la raza ibérica y el cruzamiento mayoritario con la raza duroc hace necesario también el estudio comparado de estas razas en pureza de forma que las diferencias genéticas sean más claramente detectables que en los animales híbridos.

El enorme desarrollo de las técnicas de genética molecular, como la secuenciación masiva del ARN, permite generar una enorme cantidad de información relativa a la expresión génica. Esta información, estudiada mediante programas de interpretación funcional, permite identificar genes, rutas metabólicas y factores de transcripción responsables de las diferencias observadas a nivel fenotípico entre razas, lo que ayuda a entender los mecanismos moleculares que se encuentran detrás de estas diferencias.

Paralelamente a los estudios propiamente genéticos, se han realizado esfuerzos para explorar las posibilidades que ofrece controlar la cantidad y composición de los lípidos que se incluyen en los piensos en la producción de cerdo ibérico (López Bote *et al*., 1999, 2000). Actualmente, el empleo de dietas enriquecidas en AG, especialmente en ácido oleico, es una práctica que permite mimetizar el engorde de estos animales en el sistema tradicional de montanera, basado en la ingesta de bellotas y pasto (Ventanas *et al*., 2008; Pérez-Palacios *et al*., 2009). Los trabajos previos muestran que estas estrategias permiten modificar el perfil de ácidos grasos de los diferentes depósitos grasos del cerdo ibérico (Tejeda *et al*., 2002; Ventanas *et al*., 2007; Óvilo *et al*., 2014a). Además, durante las últimas décadas se ha reconocido que determinados lípidos de la dieta tienen actividades biológicas únicas debido a sus efectos estimuladores/inhibidores sobre la transcripción de genes que codifican para enzimas que intervienen en el metabolismo lipídico (Jump, 2002; Sampath & Ntambi, 2005; Georgiadi & Kersten, 2012; Hara *et al*., 2013; Kellner *et al*., 2017). Por ello, las consecuencias del empleo de dietas enriquecidas en distintos tipos de AG puede estudiarse desde un enfoque nutrigenómico, investigando los efectos de los nutrientes sobre el fenotipo que están vehiculados por cambios en la expresión génica (Corella y Ordovas, 2009). Existen algunos trabajos recientes que muestran como esta suplementación conduce a una modificación de la composición de AG de los tejidos debido a la acumulación directa de los componentes de la dieta pero también a la modulación de la síntesis endógena mediante cambios en la expresión génica

y en la actividad enzimática de genes involucrados en la regulación de la adipogénesis, la lipogenésis y la lipolisis, modificando así la cantidad y composición de AG de los distintos tejidos y por tanto su calidad (Óvilo *et al.,* 2014a; Diaz-Rúa *et al*., 2015; Reynés *et al*., 2017).

La peculiar fisiología del cerdo ibérico y su tendencia a la acumulación grasa se deben en parte a su adaptación a la estacionalidad de los recursos alimenticios en el ecosistema de la dehesa, donde tradicionalmente se ha desarrollado (genotipo ahorrador). Es de esperar por ello, que los mecanismos moleculares de ajuste ante periodos de escasez o ayuno sean diferentes y más eficaces en el cerdo ibérico que en otras razas magras y no solo a nivel del control hipotalámico del apetito, sino también en tejidos periféricos. El estudio de las adaptaciones fisiológicas que se desencadenan como consecuencia del ayuno en distintos tejidos tiene gran interés ya que estudios recientes indican que la ingestión/ayuno de alimentos afecta la expresión de muchos factores de transcripción esenciales en la coordinación la respuesta metabólica provocada por la falta de nutrientes (Cardoso *et al*., 2017b) y que la variación genética de esos factores de transcripción afectados podría tener efectos importantes en el depósito y composición de los depósitos grasos (Ribas-Latre & Eckel-Mahan, 2016).

Pese a existir varios estudios comparando el transcriptoma de cerdos ibéricos en pureza con animales cruzados con duroc (Óvilo *et al*., 2014b; Ayuso *et al*., 2015b; Ayuso *et al*., 2016) no existen estudios que comparen ambas razas en pureza y en idénticas condiciones de manejo. Los efectos de la inclusión de distintos AG, así como la fuente de energía o la influencia de los periodos de ayuno en cerdos ibéricos y duroc en pureza, mantenidos en idénticas condiciones experimentales, tampoco han sido explorados hasta ahora. Estas aproximaciones nos proporcionarían una valiosísima información acerca de las particularidades del metabolismo de ambas razas.

Con estos antecedentes los objetivos principales de la presente Tesis Doctoral han sido:

1- Profundizar en el conocimiento de los mecanismos moleculares y los genes implicados en la regulación de la lipogénesis, lipolísis y el metabolismo lipídico así como comprender el efecto de la incorporación de distintas fuentes de energía en la composición tisular y la expresión génica en el cerdo ibérico.

Para la consecución de este objetivo se realizaron dos trabajos empleando una sola raza: cerdos ibéricos en pureza de la línea Torbiscal, orientados a mejorar la comprensión de la base molecular de la calidad de los productos del cerdo ibérico y profundizar en el conocimiento de la influencia que pueden tener las intervenciones nutricionales en su regulación. En primer lugar se estudiaron los efectos de la composición de ácidos grasos (AGS y AGPI) de la dieta de cerdos ibéricos en fase de cebo sobre el perfil de AG y sobre la expresión de genes codificantes para enzimas clave relacionadas con la adipogenesis, lipogenesis y el metabolismo lipídico, en los tejidos adiposo, muscular y hepático (Capitulo 1, Artículo 1º). En segundo lugar se evaluó el efecto de la fuente de energía de la dieta, comparando una dieta estándar con carbohidratos (CH) y una dieta enriquecida con un 6% de aceite de girasol alto oleico (HO), sobre la transcripción de un panel de genes candidato en tejido adiposo del jamón, en diferentes períodos de crecimiento y en situación de ayuno moderado (18h) (Capitulo 2, Artículo 2º).

2- Comprender los efectos conjuntos de la dieta y de la raza (ibérico y duroc en pureza) sobre la composición de los tejidos y la expresión génica, para conocer la influencia de las interacciones genética-nutrición sobre distintos procesos fisiológicos y metabólicos con trascendencia a nivel productivo.

Para la consecución de este objetivo se realizaron otros dos trabajos empleando las dos razas en pureza mantenidas en idénticas condiciones de manejo durante la fase de crecimiento, orientados a mejorar el conocimiento de los genes y rutas metabólicas implicadas en el efecto de la dieta así como los aspectos diferenciales de la respuesta a la dieta entre las dos razas estudiadas. En el tercer trabajo de esta tesis se estudiaron los efectos de la raza (ibérica *vs.* duroc), un período de ayuno largo (24 h), y la fuente de energía de la dieta (HO *vs.* CH), así como sus interacciones, sobre la composición del tejido adiposo y la expresión génica de factores implicados en adipogénesis, lipogénesis y lipólisis mediante un enfoque de genes candidato (Capitulo 3 Artículo 3º). Por último, en el cuarto trabajo de esta tesis se realizó un abordaje más ambicioso mediante la secuenciación masiva del transcriptoma para profundizar en las rutas metabólicas y redes génicas afectadas por el efecto de la dieta y la raza, así como su interacción, en la grasa subcutánea del jamón (Capitulo 4 Artículo 4º).

La comprensión de los mecanismos moleculares que favorecen el alto potencial de deposición de grasa así como el peculiar metabolismo de cerdos ibéricos en comparación con otras razas, incluso en situaciones adversas de restricción calórica o de ayuno y con distinta fuente de energía en la dieta tiene un enorme interés a nivel productivo, científico y biomédico.

3. RESULTADOS

3. CAPITULO 1

Efectos de la saturación de la grasa de la dieta sobre la composición de ácidos grasos y la transcripción génica en distintos tejidos de cerdos ibéricos

Effects of dietary fat saturation on fatty acid composition and gene transcription in different tissues of Iberian pigs.

Benítez R., Núñez Y., Fernández A., Isabel B., Fernández A.I., Rodríguez C., Barragán C., Martín-Palomino P., López-Bote C., Silió L. and Óvilo C.

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Effects of dietary fat saturation on fatty acid composition and gene transcription in different tissues of Iberian pigs

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ABSTRACT

The effect of two diets, respectively enriched with SFA (*S*) and PUFA (*P*), on FA tissue composition and gene expression was studied in fattened Iberian pigs. The FA composition of adipose, muscular and liver tissues was affected by dietary treatment. *S* group showed higher MUFA and MUFA/SFA ratio and lower PUFA and $n-6/n-3$ ratio than *P* group in all analyzed tissues. In muscle and liver the extracted lipids were separated into neutral lipids and polar lipid fractions which showed significantly different responses to the dietary treatment, especially in liver where no significant effect of diet was observed in NL fraction. The expression of six candidate genes related to lipogenesis and FA oxidation was analyzed by qPCR. In liver, *stearoyl CoA desaturase (SCD)*, *acetyl CoA carboxylase alpha (ACACA)* and *malic enzyme 1* (*ME1)* genes showed higher expression in *S* group. *SCD*, *ACACA*, *ME1*, and *fatty acid synthase (FASN)* gene expression levels showed a wide variation across the tested tissues, with much higher expression levels observed in adipose tissue than other tissues. Tissue FA profile and gene expression results support the deposition of dietary FA, the lipogenic effect of dietary saturated fat in liver and the employment of saturated dietary fat for endogenous synthesis of MUFA in all the analyzed tissues.

1. Introduction

Besides fat quantity, fatty acid (FA) composition of muscle and adipose tissues determines sensorial, technological and nutritional aspects of meat influencing its perception by the consumers (Webb & O'Neill, 2008; Wood et al., 2008). From a nutritional point of view, medical recommendations are now shifting from the reduction of fat intake towards increasing fat quality in order to maintain cardiovascular health. Meat is a primary source of dietary fat and especially of saturated fatty acids (SFA). High consumption of SFA has been associated with obesity, high plasma cholesterol and cardiovascular diseases (Chizzolini, Zanardi, Dorigoni, & Ghidini, 1999; Katan, Zock, & Mensink, 1994), while replacing SFA with MUFA or PUFA reduces the risk of coronary heart disease (de Lorgeril & Salen, 2012). At the same time, long-chain polyunsaturated fatty acids (PUFA) have been implicated in the prevention of different diseases (Nguyen, Nuijens, Everts, Salden, & Beynen, 2003; Wood et al., 2003), although nutritionists tend to focus more on the PUFA/SFA ratio and the ratio $n-6/n-3$ rather than the content of particular FAs (Jimenez-Colmenero, Ventanas, & Toldra, 2010). Although producers and consumers differ about the importance of animal FA profile in meat quality (Webb & O'Neill, 2008), an increasing number of consumers prefer meat products with higher ratios of PUFA and MUFA relative to SFA and with favorable balance between $n-6$ and $n-3$ PUFA, because of their beneficial effects on disease prevention (Kallas, Realini, & Gil, 2014; Wood et al., 2003). Hence, there has been much interest in finding ways to manipulate the FA composition of meat in order to produce functional foods (Coates, Sioutis, Buckley, & Howe, 2009). Different aspects such as feeding system, age, sex or the genetic type influence this composition, dietary manipulation of the FA profile being the most effective procedure of altering the fat composition of pig meat products (Kouba, Enser, Whittington, Nute, & Wood, 2003; Morel, McIntosh, & Janz, 2006).

In monogastric meat animal species most dietary fatty acids are absorbed directly, unchanged from the intestine (Enser, Richardson, Wood, Gill, & Sheard, 2000), and deposited in muscle and adipose tissues. Moreover, tissue fatty acid composition is also dependent on endogenous synthesis which may be also influenced by dietary composition. Changes in dietary fat have different impacts on the expression of genes related to lipid metabolism (Jump et al., 2005). Most studies have been performed in rodents, in which PUFA and SFA enriched diets have been shown to alter the transcription of genes related to

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3.1.2. INTRODUCTION

Besides fat quantity, fatty acid (FA) composition of muscle and adipose tissues determines sensorial, technological and nutritional aspects of meat influencing its perception by the consumers (Webb and O'Neill., 2008; Wood *et al*., 2008). From a nutritional point of view, medical recommendations are now shifting from the reduction of fat intake towards increasing fat quality in order to maintain cardiovascular health. Meat is a primary source of dietary fat and especially of saturated fatty acids (SFA). High consumption of SFA has been associated with obesity, high plasma cholesterol and cardiovascular diseases (Katan *et al*., 1994; Chizzolini *et al*., 1999), while replacing SFA with MUFA or PUFA reduces the risk of coronary heart disease (de Lorgeril *et al*., 2012). At the same time, long-chain polyunsaturated fatty acids (PUFA) have been implicated in the prevention of different diseases (Nguyen *et al*., 2003; Wood *et al*., 2003), although nutritionists tend to focus more on the PUFA/SFA ratio and the ratio n-6/n-3 rather than the content of particular FA (Jiménez-Colmenero *et al*., 2010). Although producers

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Some previous studies have also shown the effects of SFA and PUFA enriched diets on composition and gene transcription in different pig breeds, with no consistent results (Mitchaothai *et al*., 2007; Duran-Montge *et al*., 2009a and 2009b; Iyer *et al*., 2012). Thus, there is no clear understanding on how the dietary FA composition has an impact on different tissues, their relative influence on the direct deposition of FA or on its endogenous synthesis. Moreover, knowledge of their effects on the Iberian pig breed is scarce. Iberian breed is characterized by its high lipogenic potential

and specific tissue FA profile which is conditioned by its feeding system and by its own metabolism (López-Bote *et al*., 1998). In this sense, the breed shows a high desaturation capacity (Daza *et al*., 2005; Rey *et al*., 2006; Ventanas *et al*., 2008; Pérez-Palacios *et al*., 2009) and presents high levels of monounsaturated fatty acids (MUFA) in fat tissues. In these fatty pigs, the energy and fat metabolism could be differentially affected by diet composition with respect to lean-type breeds (Barea *et al*., 2013). Our previous work has shown that enrichment of diet with MUFA influences tissue composition but has minor influence on gene transcription in Iberian pigs, at least in the long term (Óvilo *et al*., 2014a). According to our previous results, the effects of oleic acid supplementation on candidate gene transcription seem to be more important in adipose than in other tissues.

Therefore, the objective of this study was to evaluate the effect of two diets, respectively enriched with SFA and PUFA, on FA tissue composition and gene expression of six candidate genes involved in lipogenesis and FA metabolism in adipose, muscular, hepatic and cardiac tissues of fattening Iberian pigs. Gene expression differences between tissues were also studied.

3.1.3. MATERIAL AND METHODS

Animals

The current study was carried out at the facilities of the CIA Dehesón del Encinar (Toledo, Spain), under a Project License from the INIA Scientific Ethic Committee. Animal manipulations were performed according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation.

The study comprised 27 barrows born in 15 contemporary litters of the Iberian Torbiscal strain, which were fed a barley-wheat bran-soybean meal-based diet during a pre-experimental period of 14 weeks. When pigs reached 60 kg (SD = 4 kg) of body weight (BW) they were randomly allotted to two groups, with full-sibs being split into the two groups, and were fattened with two different isocaloric and isoproteic diets. The crude energy content was 3,100 Kcal per kg of feed for both diets. Each experimental diet was respectively enriched in saturated FA (group S, n=13), with the inclusion of 5% hydrogenated lard, and polyunsaturated FA (group P, n=14), with the inclusion of 5% sunflower oil. Feed composition is shown

Table 1. Ingredients, calculated chemical composition[1] and analyzed fatty acid composition of the experimental diets. (g/kg; as-fed basis)

Diet	Saturated (S)	Polyunsaturated (P)
Ingredients		
Barley	688.7	688.7
Sunflower meal, 30% CP[2]	121.7	121.7
Soyabean meal, 44% CP	86.8	86.8
Wheat bran	25.5	25.5
Hydrogenated fat	50.0	-
Sunflower oil	-	50.0
Sodium choride	3.0	3.0
Calcium carbonate	7.0	7.0
Bicalcium phosphate	13.0	13.0
Lysine (50%)	1.3	1.3
Vitamin and mineral premix	3.0	3.0
Chemical composition, g/kg of feed		
Moisture	94.4	94.4
Lipids	67.1	68.6
Crude protein	157.0	157.0
Crude fiber	66.5	66.5
Nitrogen-free Extractives	360.4	360.4
Ash	50.9	50.9
Main Fatty acids, g/kg of feed		
C14:0	0.9	0.2
C16:0	40.4	11.5
C18:0	4.1	3.1
C18:1*n-9*	29.2	24.4
C18:2*n-6*	13.7	50.8
C18:3*n-3*	0.7	1.3

[1] According to Fundación Española Desarrollo Nutrición Animal (2010)
[2] CP: Crude Protein

in (Table **1**). During this fattening period, the pigs were raised outdoors in two separated fenced areas. Animals were manually fed twice a day and water was provided ad libitum. One long feeder was available for each experimental group (80cm/animal), which allowed all animals to eat at the same time. The average daily ration increased from 1.5 to 3.53 kg along 25 weeks until the pigs reached

150 kg (SD =12 kg) of BW. Animals were then stunned and slaughtered at a local slaughterhouse (Alcaudete de la Jara, Toledo, Spain). After slaughter, carcasses were scalded and eviscerated. Carcass weight was recorded and backfat thickness was measured on the left side of each carcass at the level of the last rib, using a vernier caliper. Tissue samples were collected from backfat, longissimus thoracis muscle, heart (left ventricle) and liver (right lateral lobe) and stored at -80ºC. The time elapsed since the stunning of the animals to the samples storing did not exceed 3 hours. Backfat samples and longissimus muscle samples were taken at the level of the last rib. Backfat samples were separated into outer and inner layers, which were separately analyzed for fatty acid composition.

Tissue and feed FA composition analyses

Lipid extracts from subcutaneous fat (inner and outer layers), were extracted by the procedure proposed by Bligh & Dyer (1959) whereas the extracted lipids from longissimus muscle and liver were separated into neutral lipids (NL) and polar lipids (PL) using aminopropyl minicolumns, following the method used by Segura & López-Bote (2014). Fat extracts were methylated in the presence of sulphuric acid and analyzed by gas chromatography as described elsewhere (López-Bote *et al*., 1997b) using a Hewlett Packard HP-6890 (Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and capillary column (HP-Innowax, 30 m x 0.32 mm i.d. and 0.25 µm polyethylene glycol-film thickness). A temperature program of 170 to 245° C was used. The injector and detector were maintained at 250° C. The carrier gas (helium) flow rate was 2 ml/min. Results were expressed as grams per 100 grams of detected FAMEs.

Dietary FA were extracted and quantified by the one-step procedure as described by Sukhija & Palmquist (1988) in lyophilized samples. Pentadecanoic acid (C15:0) (Sigma, Alcobendas, Madrid, Spain) was used as internal standard. Previously methylated FA samples were identified by gas chromatography as described above. Results were expressed as grams per Kg of feed.

Candidate gene expression analyses by quantitative PCR (qPCR)

For the candidate gene expression quantification different samples obtained from carcasses were used: a) Backfat (inner layer), b) longissimus muscle,

c) cardiac muscle and d) liver samples. For each tissue 50-100 mg samples from the 27 animals in the study were used for total RNA extraction using RiboPureTM RNA isolation kit (Ambion, Austin, USA) following the manufacturer´s recommendations. RNA obtained was quantified using a NanoDrop ND-1000 equipment (NanoDrop Technologies, Wilmington, USA) and RNA quality was assessed with an Agilent bioanalyzer device (Agilent Technologies, Palo Alto, USA). The RNA Integrity Number (RIN) values obtained were in the range 7.0 to 8.5 for all tissues, thus assuring the homogeneity and high quality of samples. First-strand cDNA synthesis was carried out with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl containing 1 µg of total RNA and following the supplier's instructions.

Six genes with functional involvement in lipogenesis and FA metabolism were selected for gene expression quantification in several tissues: stearoyl-CoA desaturase (*SCD*), fatty acid synthase (*FASN*), malic enzyme 1 (*ME1*), acetyl-CoA carboxylase alpha (*ACACA*), carnitine palmitoyltransferase1B (*CPT1*) and hydroxyacyl-CoA dehydrogenase (*HADH*).

Four lipogenic genes were studied: *SCD* encodes an endoplasmic reticulum enzyme that catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids (Bernert and Sprecher., 1977; Paton *et al*., 2009), *FASN* encodes a large multienzyme that catalyzes all steps of fatty acid synthesis (Smith *et al*., 2003; Maier *et al*., 2008), *ME1* encodes a cytosolic, NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis (Wise and Ball., 1964; Stelmańska *et al*., 2007) and *ACACA* encodes a complex multifunctional enzyme system, which catalyzes the first committed step in the biosynthesis of long-chain fatty acids by converting acetyl-CoA into malonyl-CoA (Wakil *et al*., 1983; Gallardo *et al*., 2009).

Two genes involved in β-oxidation were studied in cardiac and longissimus muscles. These are *CPT1* and *HADH*, which are involved in the transport and mitochondrial degradation of fatty acids. In longissimus we also analyzed the four lipogenic enzymes, and in cardiac muscle we studied the β-oxidation genes *CPT1* and *HADH* plus *SCD* gene, because *ACACA, FASN* and *ME1* could not be quantified in this tissue due to low expression levels.

The expression of the six candidate genes was quantified by qPCR. Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL sequences, covering

Table 2. Primer design for qPCR efficiencies (eff) in four analyzed tissues

Gene symbol	Forward primer sequence	Reverse primer sequence	Size (bp)	Adipose eff (%)	Muscle eff (%)	Liver eff (%)	Cardiac eff (%)
SCD	TCCCGACGTGGCTTTTTCTTCTC	CTTCACCCCAGCAATACCAG	205	91.7	99	97.5	92.9
FASN	GCAGGCGCGTGATGGGAATGGTG	GCCCGAGCCCGAGTGGATGAGCA	206	95.8	99	82.8	-
ACACA	CTGAGAGCTCGTTTGAAGGAATA	TTTACTAGGTGCAAGCCAGACAT	281	92	96	88.7	-
ME1	GCCGGCTTTATCCTCCTCT	TCAAGTTTGGTCTGTATTTTCTGG	223	85.4	93.8	93.8	-
CPT1	GGCGCCGCGTGAAAGCAGAC	CGCCCGCGATCATGTAGGAGAC	321	-	86.2	-	92.7
HADH	GCGGCCTCGGCAAGAAAAT	CCGTGCTGGTGGATATGCTGCTCA	259	-	90.1	-	90.6

different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in (**Table 2**). Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Takara-Bio, Otsu, Shiga, Japan) in a MX3000P (Stratagene, La Jolla, CA, USA). The qPCR reactions were prepared in a total volume of 20 µl containing 2.5 µl of cDNA (1/20 dilution), 10 µl of SYBRGreen mix, 0.4 µl of ROX II reference dye and 0.15 µM of both forward and reverse primers. As negative controls, mixes without cDNA were performed. Cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C (15 sec) and 60°C (1 min), where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95°C (15 sec) followed by 60°C (20 sec) and ramp up to 95°C with acquired fluorescence during the ramp to 0.01°C/sec. Data were analyzed using MxPro software (Stratagene La Jolla, CA, USA). All points and samples were run in triplets as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of each gene. PCR efficiencies were estimated for each analyzed gene by standard curve calculation using four points of cDNA serial dilutions. Mean Ct values were transformed to quantities using the comparative Ct method, setting the highest relative quantities for each gene to 1 (Qty=10-ΔCt/slope). Data normalization was carried out using the two most stable

endogenous genes out of: GAPDH, B2M, TBP and ACTB depending on the tissue. Endogenous genes stability measures (M) were calculated from Genorm software (Vandesompele et al., 2002). GAPDH and B2M genes were finally used for the normalization within tissue.

Also gene expression differences due to tissue effect (backfat, longissimus muscle and liver) were calculated for *SCD, ACACA, FASN* and *ME1* genes. Data normalization was carried out using *ACTB* and *TBP* genes, which showed maximum stability across tissues. For this comparison we used cDNA pools of four samples (two from each treatment).

Statistical analyses of tissue composition and candidate gene expression data

The influence of diet on fatty acid composition was separately analyzed for each fatty acid and tissue with a linear model fitting the mean, dietary treatment and residual effects. All the analyses were performed using the GLM procedure of SAS 9.1 (SAS Institute Inc., Cary, NC, USA).

Statistical analysis of gene expression data was carried out following the method proposed by Steibel *et al*. (2009) which consists of the analysis of cycles to threshold values (Ct), for the targets and endogenous genes using a linear mixed model. The following model was used for analyzing the joint expression of the target and control genes in different tissues:

$$\boldsymbol{y_{gijkr} = TG_{gi} + L_{gj} + B_{gijk} + D_{ijk} + e_{gijkr}}$$

Where $y_{gijkr} = -log_2\left(E_{gijkr}^{-Ct_{gijkr}}\right)$, E brings the efficiency of the PCR of each gene in each tissue, Ct is the value obtained from the thermocycler software for the gth gene, for the rth well of the kth animal fed with the ith dietary treatment, TG_{gi} is the specific effect of the ith dietary treatment on the expression of gene g, L_{gj} and B_{gijk} are specific random effects of the jth full-sib family and the kth pig on the expression of gene g, D_{ijk} is a random sample-specific effect common to all genes, and e_{gijkr} is a residual effect.

To test differences and interactions between classes in the expression rate of genes of interest ($diff_{TG}$) normalized by the endogenous genes, different contrasts were performed between the appropriate estimates of TG levels. Significance of $diff_{TG}$

estimates was determined with the t statistic. To obtain fold change values (FC) from the estimated $diff_{TG}$ values, the following equation was applied: $\mathrm{FC} = 2^{-diff_{TG}}$

Asymmetric (95%) confidence intervals were calculated for each FC value by using the standard error (SE) of the estimated difference:

95% confidence interval from $2^{(-diff_{TG}+1.96xSE)}$ to $2^{(-diff_{TG}+1.96xSE)}$

Gene-expression data in different tissues were analyzed entering the effects of treatment and tissue as fixed effects. Significant differences were established by Tukey-Kramer HSD post-hoc contrasts and were assumed to be statistically significant for $P \leq 0.05$. The qPCR expression data were normalized using normalization factors calculated from the endogenous genes expression values using Genorm software (Vandesompele *et al*., 2002).

3.1.4. Results and discussion

Effect of fatty acid composition of the diet on animal phenotype and tissue composition.

The two dietary groups showed similar weights throughout the experiment. Also, at slaughter, the body weight (152.5 kg vs 148.5 kg of mean BW for P and S groups, respectively) and backfat thickness (22.9 mm vs 21.1 mm for P and S groups, respectively) were not significantly different between them. This is in agreement with previous studies reporting no effect of dietary fat source or saturation on growth performance and carcass characteristics in pigs (Kouba *et al*., 2003; Morel *et al*., 2006; Olivares *et al*., 2010).

The FA profile was studied in three different tissues: subcutaneous backfat (inner and outer layers), muscle (longissimus) and liver. FA composition of subcutaneous fat showed significant differences between dietary groups (**Table 3**). Statistical analyses indicated only small differences in the response of the two backfat layers to the dietary treatment, with none of them being significantly different. In both layers, main diet effects were observed on SFA, MUFA and PUFA contents with higher MUFA and SFA in S group and higher PUFA in P group, as could be expected. The higher PUFA content in P group was mainly due to an increase in linoleic acid (2.9-fold) which was the main FA provided in the P diet. Other PUFAs with smaller percentages were also increased in P group, including linolenic acid (C18:3n-3), representative of n-3 series. The ratio MUFA/SFA was

Table 3. Least-squares means and standard errors of FA percentages and indexes in outer and inner layers of subcutaneous fat samples obtained at slaughter from Iberian pigs fed with Saturated (*S* diet) *n*=13 and Polyunsaturated (*P* diet) *n*=14.

Backfat layer	Outer layer			Inner layer		
Fatty acid[1]	S	P		S	P	
	Mean (SEM)	Mean (SEM)	*p*-value	Mean (SEM)	Mean (SEM)	*p*-value
C14:0	1.36 (0.02)	1.20 (0.02)	<.0001	1.35 (0.03)	1.20 (0.03)	0.0007
C16:0	24.62 (0.14)	21.86 (0.13)	<.0001	25.70 (0.21)	22.82 (0.19)	<.0001
C16:1n-9	0.24 (0.01)	0.23 (0.01)	0.25	0.21 (0.01)	0.20 (0.01)	0.75
C16:1n-7	2.14 (0.05)	1.62 (0.05)	<.0001	1.74 (0.07)	1.29 (0.07)	0.0003
C17:0	0.32 (0.01)	0.29 (0.01)	0.22	0.31 (0.01)	0.28 (0.01)	<.0001
C17:1	0.35 (0.01)	0.25 (0.01)	<.0001	0.28 (0.01)	0.20 (0.01)	<.0001
C18:0	11.85 (0.19)	10.88 (0.17)	<.0001	15.02 (0.32)	13.80 (0.30)	<.0001
C18:1n-9	46.13 (0.24)	39.51(0.22)	<.0001	44.30 (0.22)	37.89 (0.21)	<.0001
C18:1n-7	3.50 (0.06)	2.66 (0.06)	<.0001	2.95 (0.07)	2.22 (0.06)	<.0001
C18:2n-6	6.90 (0.19)	18.22 (0.17)	<.0001	5.66 (0.20)	16.86 (0.19)	<.0001
C18:3n-3	0.45 (0.01)	0.57 (0.01)	<0.001	0.33 (0.01)	0.49 (0.01)	<.0001
C20:0	0.08 (0.02)	0.13 (0.02)	0.32	0.20 (0.01)	0.21 (0.01)	0.20
C20:1n-9	1.35 (0.04)	1.09 (0.04)	0.0002	1.32 (0.04)	1.11 (0.04)	0.0004
C20:3n-6	0.42 (0.02)	1.09 (0.02)	<.0001	0.33 (0.02)	1.02 (0.02)	<.0001
C20:4n-6	0.14 (0.01)	0.25 (0.01)	<.0001	0.11 (0.01)	0.23 (0.01)	<.0001
SFA2	38.35 (0.26)	34.47 (0.24)	<.0001	42.72 (0.35)	38.43 (0.32)	<.0001
MUFA3	53.73 (0.29)	45.37 (0.27)	<.0001	50.82 (0.29)	42.94 (0.27)	<.0001
PUFA4	7.91 (0.20)	20.14 (0.18)	<.0001	6.45 (0.23)	18.62 (0.21)	<.0001
C18:2/C18:3	15.33 (0.20)	31.96 (0.18)	<.0001	17.15(0.21)	34.40 (0.20)	<.0001
MUFA/SFA	1.40 (0.01)	1.31 (0.01)	0.0002	1.19 (0.01)	1.11 (0.01)	0.01
PUFA/SFA	0.20 (0.01)	0.58 (0.01)	<.0001	0.15 (0.01)	0.49 (0.01)	<.0001
n-6/n-3	15.76 (0.43)	32.28 (0.40)	<.0001	17.25(0.49)	34.89(0.45)	<.0001
C18:1n-9/ C18:0	3.90 (0.06)	3.64 (0.05)	0.09	2.97 (0.08)	2.77 (0.08)	0.0031

[1]Fatty acid composition is expressed as percentage (wt/wt) of total fatty acids

[2, 3, 4] SFA (C14:0+C16:0+C18:0+C20:0). MUFA (C16:1n-7+C18:1n-9). PUFA (C18:2+C18:3+ C20:3n-9+C20:4n-6) = sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively.

higher in S group and the ratio PUFA/SFA was higher in P group (**Table 3**). Higher total MUFA content and MUFA/SFA ratio in pigs fed the S diet are mainly a consequence of the higher oleic acid content, major representative of MUFAs, in the tissues of animals in S group. The desaturation index C18:1n-9/C18:0, which is considered an indirect measure of SCD activity (Attie *et al*., 2002), was significantly higher in S group. The ratio n-6/n-3 was higher in P group, in agreement with higher diet levels of linoleic acid which is the main representative of n-6 FA. According to Wood *et al* (2003), the risk of cancer and coronary heart disease is reduced when PUFA/SFA in the diet is greater than 0.4, and the n-6/n-3 ratio is less than 4.0. In this sense, P diet improves PUFA/SFA ratio, surpassing the 0.4 threshold (in outer layer, 0.58 vs 0.20 for P and S diets, respectively). But this improvement is due to a major increase in linoleic acid, thus showing an unfavorable effect on n-6 content and n-6/n-3 ratio. On the other hand, S group shows higher MUFA and MUFA/SFA content.

For muscle and liver tissues, the extracted lipids were separated into neutral lipids (NL) and polar lipids (PL). No significant difference was observed between diets in the total amount of fat in NL and PL fractions neither in liver nor in muscle. The fatty acid profiles of both tissues were affected by dietary treatment (**Tables 4** and **5**, respectively). In both muscle and liver, the comparison between both fractions indicates significant differences of most of the fatty acids and ratios measured between the two lipid fractions. In muscle, the NL is the fat storage fraction and showed much higher SFA and MUFA and lower PUFA in comparison to the PL fraction ($P<0.0001$). However, in liver, the NL fraction showed much higher MUFA, but lower SFA and PUFA than PL fraction. These results are in agreement with previous findings in studies in Iberian pigs (Óvilo et al., 2014a) and other breeds (Kouba et al., 2003; Wood et al., 2004).

Also, several significant fraction x diet interactions were observed in both tissues, some of quantitative nature and others of qualitative nature. In muscle, no significant diet effect was found on SFA on any fraction (Table 4); and diet effects on MUFA and PUFA (C16:1n-7, C18:1n-9, C18:2n-6, C18:3n-3, C20:3n-6) were conditional on fraction ($P<0.0001$ for the interaction fraction x diet effect). Diet effects on C16:1n-7, C18:3n-3 and C20:3n-6 were opposite according to the fraction. For C18:1n-9 and MUFA the response to the diet was significantly of higher magnitude in the PL fraction. In contrast, for C18:2n-6

Table 4. Least-squares means and standard errors of FA percentages and indexes on intramuscular fat of longissimus muscle samples from Iberian pigs fed with Saturated (*S* diet) *n*=13 and Polyunsaturated (*P* diet) *n*=14.

Fatty acid[1]	Neutral lipids			Polar lipids		
	S	P		S	P	
	Mean (SEM)	Mean (SEM)	*p*-value	Mean (SEM)	Mean (SEM)	*p*-value
C14:0	1.44 (0.03)	1.41 (0.03)	0.28	5.42 (0.22)	5.10 (0.22)	0.31
C16:0	25.63 (0.23)	25.16 (0.22)	0.15	16.61 (0.39)	16.34 (0.37)	0.63
C16:1n-7	4.32 (0.13)	3.87 (0.13)	0.02	2.36 (0.13)	2.93 (0.13)	0.005
C16:1n-9	0.20 (0.00)	0.21 (0.00)	0.56	-	-	-
C17:0	0.21 (0.01)	0.20 (0.01)	0.72	1.33 (0.07)	1.47 (0.07)	0.15
C17:1	0.25 (0.01)	0.22 (0.01)	0.06	0.73 (0.05)	0.79 (0.05)	0.38
C18:0	11.66 (0.21)	12.01 (0.20)	0.23	6.78 (0.24)	7.68 (0.23)	<0.001
C18:1n-9	47.70 (0.39)	45.60 (0.38)	<0.001	16.80 (0.49)	7.70 (0.48)	<0.001
C18:1n-7	4.46 (0.13)	3.99 (0.12)	0.01	2.99 (0.11)	2.51 (0.11)	<0.001
C18:2n-6	2.73 (0.35)	5.72 (0.34)	<0.001	24.98 (0.60)	33.32 (0.58)	<0.001
C18:3n-3	0.16 (0.02)	0.21 (0.02)	0.04	0.41 (0.00)	0.20 (0.00)	<0.001
C18:4n-3	0.10 (0.00)	0.09 (0.00)	0.05	0.06 (0.02)	0.06 (0.02)	0.97
C20:0	0.12 (0.00)	0.13 (0.00)	0.15	0.25 (0.08)	0.17 (0.07)	0.48
C20:1n-9	0.61 (0.07)	0.53 (0.07)	0.03	0.25 (0.06)	0.21 (0.06)	0.63
C20:2n-6	0.14 (0.02)	0.28 (0.03)	<0.001	0.37 (0.04)	0.54 (0.04)	0.004
C20:3n-6	0.02 (0.00)	0.05 (0.00)	<0.001	1.02 (0.03)	0.80 (0.03)	<0.001
C20:4n-6	0.11 (0.01)	0.15 (0.01)	0.006	8.90 (0.19)	9.48 (0.18)	0.04
SFA2	39.15 (0.40)	38.96 (0.39)	0.73	30.39 (0.41)	30.78 (0.40)	0.51
MUFA3	57.58 (0.49)	54.49 (0.48)	<0.001	31.29 (0.46)	22.05 (0.45)	<0.001
PUFA4	3.27 (0.39)	6.51 (0.38)	<0.001	38.28 (0.62)	47.12 (0.59)	<0.001
MUFA/SFA	1.47 (0.02)	1.40 (0.02)	0.03	1.03 (0.02)	0.72 (0.02)	<0.001
PUFA/SFA	0.08 (0.00)	0.16 (0.00)	<0.001	1.26 (0.00)	1.53 (0.00)	<0.001
n-6/n-3	18.51 (0.61)	29.60 (0.59)	<0.001	20.56 (2.90)	34.49 (2.85)	0.002
C18:1n-9/ C18:0	4.12 (0.08)	3.85 (0.08)	0.03	2.49 (0.05)	1.00 (0.05)	<0.001

[1]Fatty acid composition is expressed as percentage (wt/wt) of total fatty acids

[2, 3, 4] SFA (C14:0+C16:0+C18:0+C20:0). MUFA (C16:1n-7+C18:1n-9). PUFA (C18:2+C18:3+ C20:3n-9+C20:4n-6) = sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively.

Table 5. Least-squares means and standard errors of FA percentages and indexes on liver samples from Iberian pigs fed with Saturated (*S* diet) *n*=13 and Polyunsaturated (*P* diet) *n*=14.

Fatty acid[1]	Neutral lipids			Polar lipids		
	S	P		S	P	
	Mean (SEM)	Mean (SEM)	*p*-value	Mean (SEM)	Mean (SEM)	*p*-value
C14:0	0.55 (0.04)	0.52 (0.04)	0.58	0.17 (0.02)	0.21 (0.02)	0.21
C16:0	16.97 (0.39)	16.83 (0.38)	0.38	15.16 (0.44)	15.17 (0.42)	0.99
C16:1*n*-9	0.61 (0.03)	0.55 (0.03)	0.16	0.16 (0.02)	0.20 (0.02)	0.04
C16:1*n*-7	1.04 (0.07)	1.04 (0.07)	0.97	0.36 (0.04)	0.34 (0.04)	0.77
C17:0	0.75 (0.04)	0.81 (0.04)	0.25	0.84 (0.05)	0.93 (0.05)	0.21
C17:1	0.29 (0.02)	0.31 (0.02)	0.38	0.22 (0.01)	0.17 (0.01)	<.0001
C18:0	22.74 (0.66)	23.35 (0.63)	0.63	31.53 (1.40)	29.42 (1.25)	0.29
C18:1*n*-9	21.99 (1.03)	21.82 (0.99)	0.90	14.75 (0.25)	10.78 (0.24)	<.0001
C18:1*n*-7	1.67 (0.06)	1.67 (0.06)	0.95	1.30 (0.04)	1.08 (0.04)	0.001
C18:2*n*-6	15.45 (1.22)	14.59 (1.18)	0.61	9.44 (0.35)	14.79 (0.34)	<.0001
C18:3*n*-3	0.36 (0.03)	0.33 (0.02)	0.28	0.23 (0.01)	0.21 (0.01)	0.19
C18:4*n*-3	0.38 (0.07)	0.29 (0.07)	0.18	-	-	-
C20:0	0.13 (0.02)	0.11 (0.01)	0.32	0.15 (0.00)	0.18 (0.01)	0.12
C20:1*n*-9	0.41 (0.02)	0.41 (0.02)	0.98	0.15 (0.01)	0.14 (0.00)	0.33
C20:2*n*-6	0.53 (0.10)	0.57 (0.10)	0.75	0.70 (0.04)	0.33 (0.04)	<.0001
C20:*3n*-6	0.58 (0.04)	0.54 (0.04)	0.54	0.45 (0.03)	0.45 (0.03)	0.99
C20:4*n*-6	12.90 (0.46)	13.27 (0.44)	0.56	19.61 (0.52)	20.85 (0.50)	0.08
SFA[2]	41.14 (0.57)	41.65 (0.55)	0.53	47.86 (0.98)	45.93 (0.95)	0.18
MUFA[3]	26.20 (1.20)	26.06 (1.17)	0.93	17.32 (0.31)	13.18 (0.30)	<.0001
PUFA[4]	32.66 (1.39)	32.26 (1.34)	0.84	34.81 (0.83)	40.87 (0.81)	<.0001
MUFA/SFA	0.64 (0.02)	0.63 (0.02)	0.77	0.36 (0.01)	0.29 (0.01)	0.003
PUFA/SFA	0.79 (0.02)	0.77 (0.02)	0.89	0.73 (0.04)	0.89(0.04)	<.0001
n-6/*n*-3	17.25 (8.23)	15.38 (7.94)	0.61	33.40 (2.36)	41.56 (2.32)	0.03
C18:1*n*-9/ C18:0	0.98 (0.07)	0.95 (0.06)	0.75	0.47 (0.02)	0.36 (0.02)	0.001

[1]Fatty acid composition is expressed as percentage (wt/wt) of total fatty acids

[2, 3, 4] SFA (C14:0+C16:0+C18:0+C20:0). MUFA (C16:1n-7+C18:1n-9). PUFA (C18:2+C18:3+ C20:3n-9+C20:4n-6) = sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively.

and PUFA, the response was bigger in NL fraction. Jointly the dietary effects on both muscle fractions can be summarized as an increase in MUFA in S group and increase in PUFA in P diet, without effects on SFA (Table 4). As in adipose tissue, PUFA/SFA muscle ratio was higher in animals fed the P diet, but n-6/n-3 ratio indicated a lesser healthy meat than that of S diet (Wood et al., 2003; Kallas et al., 2014). Also, higher MUFA, MUFA/SFA and C18:1n-9/C18:0 ratios were observed in S group, with SFA level being unaffected.

Results of the dietary effects on liver FA profile are shown in table 5, for both neutral and polar lipid fractions. Significant interaction fraction x diet were observed for most FA and indices. In fact, no significant effect of diet was observed in the NL fraction of liver, while several FA were affected in the PL fraction. In this fraction, as in muscle, no differences were observed in SFA due to diet effect. But higher MUFA, MUFA/SFA and C18:1n-9/C18:0 ratios were detected in S group while higher PUFA was detected in P group, in accordance with results in other tissues. Differences in the response of lipid fractions to dietary treatment indicate a differential regulation of structural and storage lipids, especially in liver. Sampels et al., (2011) found a higher PUFA content in the PL fraction of liver than in the NL fraction and related this fact with a preferential storage of C18:2n-6 FA in the PL fraction while C18:3n-3 is stored more evenly in both fractions (Enser et al., 2000).

Results show greater diet effects on FA profile of subcutaneous adipose tissue than on the longissimus muscle or liver. This can be explained by the fact that the percentage of intramuscular fat in the longissimus muscle is almost constant in heavy pigs whereas the backfat is greatly increasing and thus it may have a higher susceptibility to dietary modifications (D'Souza et al., 2012). Similar results have been observed in other studies of dietary fatty acid modifications (Morel et al., 2006, Apple et al., 2009). Other works have shown a more homogeneous response to diet in the different tissues, possibly related to a younger age of the sampled animals (Óvilo et al., 2014a), and others have shown tissue specific effects between different muscles (Pérez-Palacios et al., 2009). Independently of the susceptibility differences and fraction effects explained, in all analyzed tissues, the S group show higher MUFA and lower PUFA and n-6/n-3 ratio than P group. Similar results have been observed in other breeds but were only significant in adipose tissue (Duran-Montgé et al., 2009a). It is interesting to note that the

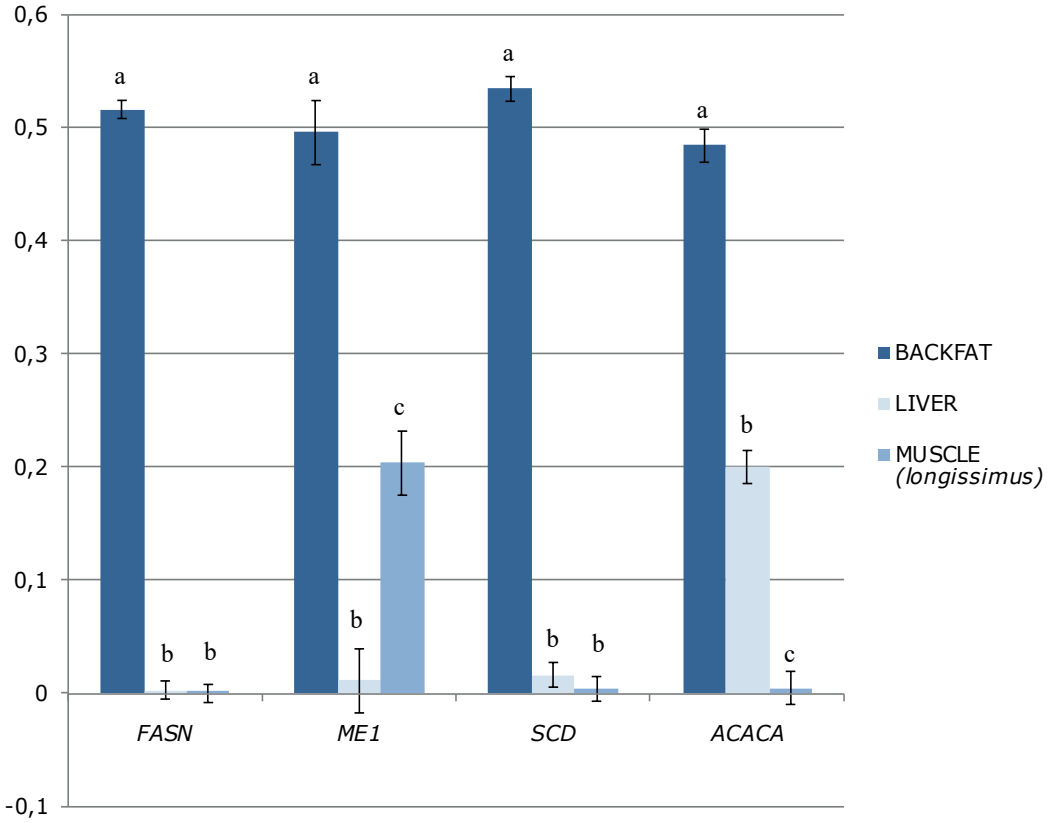
content of C18:1n-9 in the dietary supply was higher in the S group (29.2 vs 24.4 g C18:1n-9/Kg feed). Thus, the higher MUFA content observed in S group, may result in part from this difference. On the other hand, a higher desaturation of SFA may be occurring in the S group. This last hypothesis is supported by the higher MUFA/SFA and C18:1n-9/C18:0 ratios observed in S group in all tissues, and the lack of differences in SFA between diet groups observed in muscle and liver. In all tissues, the S group should be expected to show higher SFA levels, due to the direct deposition of dietary FA. The lack of a higher SFA content in S group, joint with the higher desaturation ratios observed in S samples is an indication of the tissue employment of dietary SFA for the endogenous synthesis of MUFA.

Effects of dietary fat type on candidate gene expression: Fat, liver, muscular and cardiac tissues.

Four lipogenic genes (*SCD, FASN, ME1 and ACACA*) were selected for quantification in adipose, hepatic and muscular tissues. Among them, the SCD gene was the only successfully quantified in all analyzed tissues. In contrast, FASN, ME1 and ACACA genes expressed at very low levels in cardiac tissue preventing their quantification (results not shown).

Lipogenic gene expression (*SCD, FASN, ACACA and ME1*) showed a wide variation across adipose, hepatic and muscular tissues. The results obtained for the effects of tissue on these genes expression are shown in (Figure 1). Significant expression differences were detected among all tissues in the pairwise comparisons. For *SCD, ACACA* and *FASN* genes, the highest expression was found in backfat, followed by liver and muscle. For SCD gene, backfat expression level was 31-fold higher than liver and 132-fold higher than muscle. ACACA gene expression in backfat was 2-fold higher than liver and 96-fold higher than muscle. The highest FASN expression was found in backfat even 173-fold more expression than liver and 1300-fold more expression than muscle. The low expression observed in these tissues contrast with the very high FASN expression detected in backfat. For ME1 gene, the highest expression was also detected in backfat, but a higher expression was observed in muscle than in liver, in contrast to the other three genes, with backfat showing 2.5-fold higher expression than muscle and 45.5-fold more than liver expression.

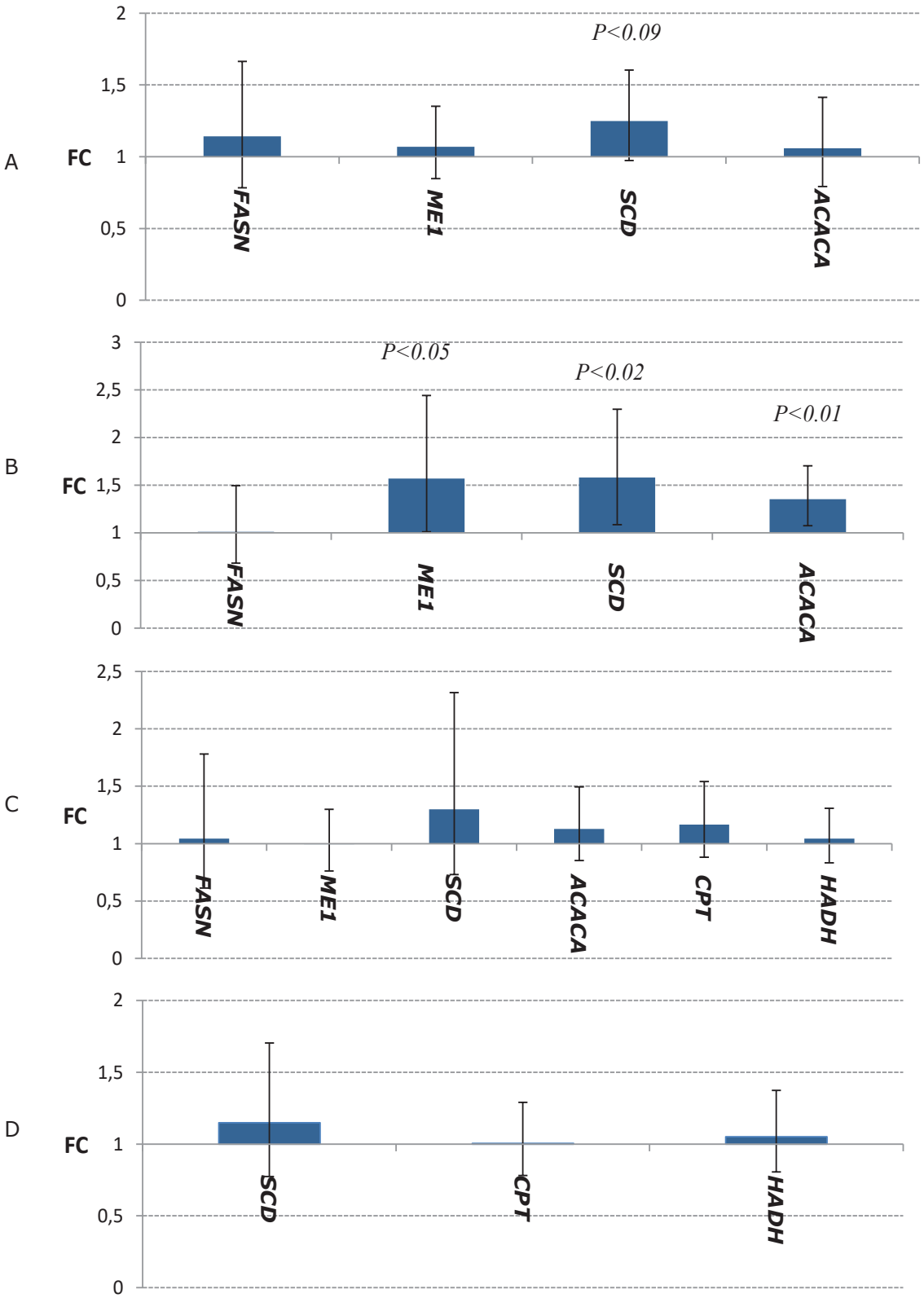
Figure 1. Candidate gene expression levels among tissues. Mean values and standard error of relative expression for FASN, ME1, SCD and ACACA genes in backfat, liver and longissimus muscle. Bars with different superscript are significantly different values (P < 0.0001).



Results of the comparison of gene expression levels between dietary groups in all studied tissues are shown in (Figure 2). In adipose tissue samples, *SCD* gene showed a trend for 1.25-fold higher expression in the S group (*P*-value≤0.09). In liver, *SCD, ACACA* and *ME1* genes showed higher expression in S group (*P*-value ≤0.02, P-value≤0.01 and P-value≤0.05, respectively). *SCD* gene showed 1.58-fold, *ACACA* gene showed 1.35-fold and ME1 showed 1.57-fold higher expression in S group than in P group. No statistically significant difference in gene expression was detected in longissimus nor cardiac muscles.

The stearoyl-CoA desaturase *(SCD*) gene is probably the most obvious candidate to be modulated by the dietary treatment applied. This gene has been proposed as a potential biomarker for fat deposition (Merino et al., 2010) and associations between its expression and intramuscular fat content in pigs have been reported (Canovas et al., 2009; Zhao et al., 2009; Estany et al., 2014). *SCD* gene expression has been shown to be downregulated in vitro in response to linoleic and oleic acids in several species (Zulkifli et al., 2010). The effect is a consequence of the

Figure 2. Fold-change (FC) ratios of candidate gene expression between Iberian pigs fed saturated vs polyunsaturated diets in (A) adipose (inner layer of backfat), (B) hepatic, (C) longissimus and (D) cardiac muscle tissues. FC values higher than 1 indicate higher expression in S group.



reduction of *SCD* promoter activity in response to the FA treatment. Specifically, the fatty acids are known to bind a highly conserved PUFA response region (PUFA-RR) of the *SCD* gene promoter, which contains several transcription factor binding sites. In vitro studies show that binding of FA to the PUFA-RR inhibits *SCD* transcription, with linoleic acid showing a more potent effect than oleic acid. In vivo, *SCD* gene expression has been observed to be influenced by the FA dietary content (and specifically by dietary oleic acid) in adipose tissue and liver, but not in semimembranosus muscle of Duroc x Landrace pigs (Duran-Montge et al., 2009b). Other tissue–specific responses of porcine *SCD* function have also been previously reported in dietary trials of protein reduction (Doran et al., 2006). Our previous results in Iberian pigs with an oleic enrichment dietary intervention do not agree with a repressing effect of oleic acid on *SCD* gene expression. No significant difference between oleic and carbohydrate dietary groups was observed on the *SCD* expression in any of the analyzed tissues (Óvilo et al., 2014a).

Nevertheless, present results show a lower *SCD* gene expression in animals fed a PUFA enriched diet, confirming previous findings supporting the inhibitory role of linoleic acid on *SCD* gene promoter in liver and adipose tissues. On the other hand, besides the inhibitory role of PUFAs on *SCD* gene promoter, an induction of *SCD* gene expression in S group could be a consequence of a higher substrate availability (C16:0 and C18:0), in agreement with previous results indicating the key role of SCD mediating the pro-lipogenic effects of dietary saturated fat (Sampath et al., 2007). In the present study, the main transcriptional response observed is an increase in *SCD* gene expression in liver and adipose tissue on S group, with 1.58-fold and 1.24-fold higher expression than P group (*P*-value≤0.02 and P-value≤0.09, respectively). These gene expression changes are consistent with the effects observed at the tissue composition level, with S group showing higher MUFA level and desaturation indexes than P group, and would provide a molecular evidence of the higher desaturation function induced by dietary SFA.

A higher expression of *ME1* and *ACACA* genes was also observed in hepatic tissue of animals fed a saturated diet. These enzymes participate in initial stages of lipogenesis. As previously indicated the expression of both genes is known to be related to nutritional issues. Most previous evidences indicate that SFA stimulates lipogenesis (Sampath et al., 2007) and PUFA upregulate genes related to fatty acid oxidation whilst downregulating genes related to lipid synthesis

(Proud et al., 2004). For example, rates of fatty acid biosynthesis are reduced in liver and adipose tissues of rats fed PUFA enriched diets, due to the effects of n-6 and n-3 PUFAs on the expression of genes *as FASN, ME1, ACACA* or *SCD* (Sessler and Ntambi., 1998). Nevertheless, in other studies lipogenic gene expression was shown to be lower in pigs fed SFA diet than those fed PUFA, suggesting that saturated FA may inhibit pig de novo fat synthesis (Duran-Montgé et al., 2009b). Our results are in agreement with previous studies indicating that dietary PUFA and increases in tissue PUFA are associated with lower expression of lipogenic enzymes, while SFA diet induces lipogenesis (Dentin et al., 2005; Sampath et al., 2007; Corominas et al., 2013).

Previous results observed in vitro and in other pig breeds and species, showed that oleic acid inhibits *SCD* expression, and high carbohydrate diets induces its expression (Zulfikli et al., 2010; Duran-Montgé et al., 2009b; Paton & Ntambi et al., 2009). In the actual work higher *SCD* gene expression is observed in the group with higher MUFA content. According to previous comparative studies performed under identical environmental and nutritional conditions, Iberian pigs are characterized by a higher MUFA synthesis compared to other lean breeds, apart from its known higher lipogenic potential (López-Bote et al., 1998: Serra et al., 1998; Barea et al., 2013; Isabel et al., 2014). The last two cited studies conclude that, besides the FA dilution effect of a higher whole fattening, Iberian pigs show higher desaturation capacity than lean pig genotypes. Also, in a recent muscle transcriptome study comparing purebred vs crossbred Duroc x Iberian piglets, phenotypic differences were observed at very early stages of development, with pure Iberian piglets showing higher MUFA content at weaning. This observation coincided with a higher *SCD* gene expression in muscle from purebred animals, thus providing a molecular basis for the higher desaturation capacity of Iberian pigs (Óvilo et al., 2014b). These metabolic and genetic singularities of the Iberian pig breed could influence some of the results reported in the present study.

Two types of muscles were studied, a skeletal muscle of high economic importance (longissimus) and cardiac muscle. It is known that mammalian organisms respond to elevations in fatty acid levels by increasing the expression in cardiac and skeletal muscles of various proteins involved in fatty acid utilization (Van der Lee et al., 2000; Young et al., 2001). Specifically, the cardiac muscle is

considered the key tissue for FA β-oxidation studies, due to its high mitochondria content (Cordero et al., 2011; Vicente et al., 2013). In our work, an excess of PUFA supply would be expected to increase the function of pathways related to transport of long fatty acids to mitochondria for being processed. In this sense, *CPT1* and *HADH* genes have been considered candidates in this study because they would be expected to increase their expression or activity in muscle in response to an excess of tissue PUFAs content. An increase in the expression or activity of this type of genes could be a mechanism to maintain the adequate proportions of FA after PUFA-enriched feeding, avoiding an excessive accumulation of linoleic acid which would result in softening of fat depots. Nevertheless, we do not detect any difference in gene expression according to dietary treatment in none of them in any analyzed muscle, thus discarding this hypothesis. On the other hand, regulation of these same enzymes at other levels cannot be discarded.

Our work agrees with previous studies reporting the modulation of gene expression by FA composition of the diet and also the tissue-specificity of this effect (Duran-Montgé et al., 2009b; Jump et al., 2005). Liver and specially adipose tissue have been proposed as the main lipogenic tissues in the pig (Duran-Montgé et al., 2009b), and thus higher influence of the diet on lipogenic genes is expected to appear in these tissues. In our work a higher transcriptional response is obtained in liver. This could be related to the differences of expression of these lipogenic genes among tissues. In liver, a more limited expression of these genes could be associated with a more regulated function. Also, the higher transcriptional response observed in liver is coincident with a lower effect of diet on FA composition, suggesting higher metabolic adaptation in this tissue faced towards the maintenance of more or less constant FA composition, as it is seen in the hepatic NL fraction, which is not modified by the diet at all.

In this work, the transcriptional response was studied in a single time-point and for a long term dietary treatment. Future studies should address the effects of diet in the short-term and in sequential analyses, because a metabolic adaptation to dietary influences could be influencing the results observed. Also, the relationship between mRNAs and their coded proteins is not linear and increases in mRNA may not coincide with increases in protein activity. Thus, more powerful studies addressing gene expression, protein and enzyme activity measurements should also be performed in different genetic backgrounds in order to clarify

the functional consequences of dietary interventions regarding their effects on metabolism. On the other hand, differences in the regulation of *SCD* gene in pure Iberian pigs in comparison to lean breeds should be explored in future works in order to understand its metabolic singularities.

3.1.5. Conclusions

Iberian pigs fed SFA-enriched diet show increased MUFA content and monounsaturation ratios in the three studied tissues and increased SFA content in adipose tissue only, indicating the employment of saturated dietary fat for endogenous synthesis of MUFA. This is in agreement with a diet-induced transcriptional response observed mainly in liver, with a higher expression of *SCD* gene in S group. Also, higher expression of hepatic *ACACA* and *ME1* genes in SFA-fed diets suggests increased lipogenesis. Gene expression results are concordant with the inhibitory role of linoleic acid on SCD gene expression and the negative effects of PUFA enriched diets on lipogenesis.

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3. CAPITULO 2

Respuesta transcripcional de genes del metabolismo lípidico en tejido adiposo de cerdos ibéricos alimentados con una dieta enriquecida en ácido oleico *versus* carbohidratos

Adipose tissue transcriptional response of lipid metabolism genes in growing Iberian pigs fed oleic acid v. carbohydrate-enriched diets.

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Adipose tissue transcriptional response of lipid metabolism genes in growing Iberian pigs fed oleic acid *v.* carbohydrate enriched diets

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Diet influences animal body and tissue composition due to direct deposition and to the nutrients effects on metabolism. The influence of specific nutrients on the molecular regulation of lipogenesis is not well characterized and is known to be influenced by many factors including timing and physiological status. A trial was performed to study the effects of different dietary energy sources on lipogenic genes transcription in ham adipose tissue of Iberian pigs, at different growth periods and on feeding/fasting situations. A total of 27 Iberian male pigs of 28 kg BW were allocated to two separate groups and fed with different isocaloric feeding regimens: standard diet with carbohydrates as energy source (CH) or diet enriched with high oleic sunflower oil (HO). Ham subcutaneous adipose tissue was sampled by biopsy at growing (44 kg mean BW) and finishing (100 kg mean BW) periods. The first sampling was performed on fasted animals, while the last sampling was performed twice, with animals fasted overnight and 3 h after refeeding. Effects of diet, growth period and feeding/fasting status on gene expression were explored quantifying the expression of a panel of key genes implicated in lipogenesis and lipid metabolism processes. Quantitative PCR revealed several differentially expressed genes according to diet, with similar results at both timings: RXRG, LEP *and* FABP5 *genes were upregulated in HO group while* ME1, FASN, ACACA *and* ELOVL6 *were upregulated in CH. The diet effect on* ME1 *gene expression was conditional on feeding/fasting status, with the higher* ME1 *gene expression in CH than HO groups, observed only in fasting samples. Results are compatible with a higher* de novo *endogenous synthesis of fatty acids (FA) in the carbohydrate-supplemented group and a higher FA transport in the oleic acid-supplemented group. Growth period significantly affected the expression of most of the studied genes, with all but* PPARG *showing higher expression in finishing pigs according to a pattern dissimilar from the usual in cosmopolitan pig breeds. Feeding/fasting status only influenced* PPARG *gene transcription. The lack of effects of feeding/fasting status on lipogenic gene expression and the higher* ME1 *response to diet in fasting samples than in postprandial sampling, suggest the persistence of* de novo *lipogenesis during fasting. Overall results improve the understanding of the influence of different factors on lipid metabolism regulation in Iberian pigs.*

Keywords: gene expression, dietary oleic acid, adipose tissue, lipid metabolism, Iberian pig

Implications

Dietary interventions influence body and tissue composition; and affect tissue metabolism by influencing gene expression. We explore the transcriptional effects of diets with different energy source and evaluate the effects of growth period and feeding/fasting on lipogenic gene expression. Results deepen in the understanding of lipogenesis regulation by nutritional factors in fatty pigs along growth, and are of interest for pig production facing with a variety of nutritional resources that can be used for diet formulation. The specific mechanisms underlying the high lipogenic and desaturation potential of Iberian pigs should be addressed in future comparative studies employing different breeds.

Introduction

Animal tissue fat content and composition are important determinants of meat quality (Wood *et al.*, 2008), which are influenced by nutritional, hormonal and metabolic factors. Dietary approaches are being essayed in order to influence

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3.2.1. ABSTRACT

Diet influences animal body and tissue composition due to direct deposition and to the nutrients effects on metabolism. The influence of specific nutrients on the molecular regulation of lipogenesis is not well characterized and is known to be influenced by many factors including timing and physiological status. A trial was performed to study the effects of different dietary energy sources on lipogenic genes transcription in ham adipose tissue of Iberian pigs, at different growth periods and on feeding/fasting situations. Twenty seven Iberian male pigs of 28 kg body weight (**BW**) were allocated to two separate groups and fed with different isocaloric feeding regimens: standard diet with carbohydrates as energy source (**CH**) or diet enriched with high-oleic sunflower oil (**HO**). Ham subcutaneous adipose tissue was sampled by biopsy at growing (44 kg mean BW) and finishing (100 kg mean BW) periods. The first sampling was performed on fasted animals, while the last sampling was performed twice, with animals fasted overnight and three hours after refeeding. Effects of diet, growth period and feeding/fasting status on gene expression were explored quantifying the expression of a panel of key genes implicated in lipogenesis and lipid metabolism processes. Quantitative PCR revealed several differentially expressed genes according to diet, with similar results at both timings: *RXRG*, *LEP* and *FABP5* genes were upregulated in HO group while *ME1*, *FASN*, *ACACA* and *ELOVL6* were upregulated in CH. The diet effect on *ME1* gene expression was conditional on feeding/fasting status, with the higher *ME1* gene expression in CH than HO groups, observed only in fasting samples. Results are compatible with a higher *de novo* endogenous synthesis of fatty acids (**FA**) in the carbohydrate-supplemented group and a higher FA transport in the oleic acid-supplemented group. Growth period significantly affected the expression of most of the studied genes, with all but *PPARG* showing higher expression in finishing pigs according to a pattern dissimilar from the usual in cosmopolitan pig breeds. Feeding/fasting status only influenced *PPARG* gene transcription. The lack of effects of feeding/fasting status on lipogenic gene expression and the higher *ME1* response to diet in fasting samples than in postprandial sampling, suggest the persistence of *de novo* lipogenesis during fasting. Overall results improve the understanding of the influence of different factors on lipid metabolism regulation in Iberian pigs.

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3.2.2. IMPLICATIONS

Dietary interventions influence body and tissue composition; and affect tissue metabolism by influencing gene expression. We explore the transcriptional effects of diets with different energy source and evaluate the effects of growth period and feeding/fasting on lipogenic gene expression. Results deepen in the understanding of lipogenesis regulation by nutritional factors in fatty pigs along growth, and are of interest for pig production facing with a variety of nutritional resources that can be used for diet formulation. The specific mechanisms underlying the high lipogenic and desaturation potential of Iberian pigs should be addressed in future comparative studies employing different breeds.

3.2.3. INTRODUCTION

Animal tissue fat content and composition are important determinants of meat quality (Wood *et al*., 2008), which are influenced by nutritional, hormonal and metabolic factors. Dietary approaches are being essayed in order to influence body and tissue composition, aiming to improve the nutritional value of pig meat and products (Raes *et al*., 2004). These approaches usually involve modifications of dietary energy content, energy source (fat vs carbohydrates) or FA composition. Regarding FA composition, diets enriched with different sources of FA have been essayed in pigs. These interventions have remarkable results for certain FA which proportions in animal tissues increase linearly as the dietary intake increase, as linoleic acid which is entirely derived from diet (Wood *et al.*, 1984). Nevertheless, other monounsaturated and saturated FA may be products of endogenous synthesis in the animal, and interconversions between them limit the impact of dietary additions (Wood *et al.*, 2008).

The failure or inconsistency of nutritional approaches to modulate fat deposition and to influence fat composition has raised the interest in the molecular regulation of lipogenesis. Nutrients influence metabolism by affecting gene expression of lipid metabolism enzymes and thus the balance between lipogenesis and lipolysis/FA oxidation. This balance determines the net composition of fat tissues. Lipogenesis is well known to be stimulated by high carbohydrate (CH) diet (Kersten, 2001), mainly postprandially, as plasma glucose levels stimulate lipogenesis by different mechanisms. On the opposite, lipogenesis is inhibited by polyunsaturated

fatty acids (**PUFA**), high fat diets and food deprivation (Sampath and Ntambi, 2006; O´Hea and Leveille, 1969; Kersten, 2001), and the latter one conversely enhances lipolysis in adipose tissue (Viscarra and Ortiz, 2013). These effects are in part mediated by hormones as leptin (Wang *et al.*, 1999), which stimulates FA oxidation and inhibits lipogenesis by down-regulating the expression of genes involved in FA and triglycerides synthesis. Impacts of diet composition on the expression of genes related to lipid metabolism are known to be highly dependent on different factors, including the species, age, tissue and treatment length and timing (Ding *et al*., 2003; Duran-Montgé *et al*., 2009). Regarding timing, many transcripts have transient changes in response to a stimulus (Ding *et al*., 2003) and thus short term and serial studies are indicated to understand the diet effects on tissue metabolism and gene expression.

The traditional fattening system of Iberian pigs is based on the intake of acorns in the finishing growth period, which provide high levels of monounsaturated fatty acids (**MUFA**), mainly oleic acid (López-Bote, 1998). This production system is the basis of highest-quality dry-cured pig products, and a reference model for sustainable production of many local Mediterranean breeds (Pugliese and Sirtori, 2012). In Iberian pigs, higher leptin levels than other lean breeds are observed, concomitantly with a very high lipogenic potential (López-Bote, 1998; Fernández-Figares *et al.*, 2007). This pattern is in agreement with the *leptin resistance* syndrome proposed as cause of extreme fattening characteristic of this breed. Besides, the Iberian breed is characterized by a specific tissue FA profile and a high desaturation capacity (Barea *et al.*, 2013). MUFA enriched diets, through the inclusion of high oleic acid sunflower oil, are being used in Iberian pigs intensive fattening systems in order to mimic the FA profile characteristic of pigs fattened with the traditional system. The effects of these diets on the FA composition of different tissues have been studied in the last decade, reporting non-consistent and tissue-specific effects (Pérez-Palacios *et al*., 2009). Recently, the impact of high oleic sunflower oil (HO) vs CH enriched diets on tissues composition joint with their long term effects on gene transcription have been also evaluated (Óvilo *et al.*, 2014). According to this study, Iberian pigs fed the HO diet showed higher MUFA content and lower saturated fatty acids (**SFA**) in all the analyzed tissues. On the other hand, animals receiving CH diet were able to synthesize considerable amounts of SFA but also MUFA. Nevertheless, the effect of diet

on the transcription of lipogenic enzyme genes was modest in the long term, suggesting a subtle effect of the diet on the regulation of several lipid metabolism pathways. These joint results suggest that *de novo* lipogenesis coupled with elongation and desaturation may be playing a key role in the tissue FA profiles, and these pathways may be differentially regulated between dietary groups. The timing when samples are obtained for gene transcription analysis may be also of importance. Indeed, changes in lipogenesis along growth (Anderson and Kaufman, 1973; Hood and Allen, 1973; Mourot *et al.*, 1996) may influence the impact of diet on lipogenesis at different growth periods, thus, short- and long-term effects must be explored. Short-term effects are especially interesting in this fatty pig breed in which adipo and lipogenesis processes are expected to start more precociously than in other selected lean breeds (López-Bote, 1998).

The present work employed the same experimental material studied previously in Óvilo *et al*. (2014), with the objective of evaluating short and long term effects of diet energy source (HO vs CH) on transcription of candidate genes in ham subcutaneous adipose tissue of Iberian pigs. Also, effects on gene expression of the growth period and the feeding/fasting status of the animals were explored.

3.2.4. MATERIAL AND METHODS

Animals

The current study was carried out at the facilities of the CIA Deheson del Encinar (Toledo, Spain). Animal manipulations were performed in compliance with the regulations of the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 about the protection of animals used in experimentation. The study comprised 27 barrows born in 15 litters of the Torbiscal Iberian strain. At 28 kg (standard deviation, **SD** = 3 kg) of body weight (BW) the animals were distributed in two dietary groups (n = 14 and 13, respectively), and penned individually, with full-sibs pairs being splitted in the two groups. A group was fed with a high-oleic sunflower oil enriched diet (HO) and the other one (CH) with a diet of carbohydrates as the main source of energy. Feeds composition is shown in Table 1. The energy content per kg of feed was 9.6 MJ of net energy for the CH diet, and 10.43 MJ for the HO diet. During the 23 weeks of treatment, animals were fed individually twice a day according

Table 1. Ingredients, calculated chemical composition[1] and fatty acid composition of the experimental diets (g/kg; as-fed basis)

Diet	Carbohydrate (CH)[2]	High oleic (HO)[3]
Ingredients, g/kg of feed		
Barley	470.80	479.96
Wheat	450.00	250.00
Soybean meal, 44% CP[4]	53.15	107.69
Bicalcium phosphate	7.50	9.77
Calcium carbonate	5.74	3.02
Soybean hulls	2.75	30.03
Sodium choride	4.00	4.00
Vitamin and mineral premix[5]	3.00	3.00
Lysine	3.06	2.53
High oleic sunflower oil	0.00	60.00
Lucern meal, 15% CP	0.00	50.00
Chemical composition[1], g/kg of feed		
Moisture	105	96
Lipids	19	78
Crude protein	129	142
Crude fiber	38	58
Ash	37	44
Main Fatty acids, g/kg of feed		
C14:0	0.03	0.20
C16:0	2.19	5.85
C18:0	0.12	2.46
C18:1 n-9	1.52	44.78
C18:2	5.97	15.54

[1] According to Fundación Española Desarrollo Nutrición Animal (2010)
[2]CH = carbohydrate diet without added fat.
[3]HO = high oleic diet with high oleic sunflower oil.
[4]CP: crude protein
[5] Vitamin-mineral premix provided per kg of feed: Vitamin D3, 1,200 IU; Vitamin E, 11 UI; Vitamin B1, 0.2 mg; Vitamin B2, 2.5 mg; vitamin B12, 0.015 mg; Vitamin B6, 1.3 mg; Calcium pantothenate, 8 mg; Nicotinic acid, 20 mg; Biotin, 0.1 mg; Folic acid, 0.5 mg; Vitamin K3, 2 mg; Fe, 75 mg; Cu, 1 6.0 mg; Co, 0.10 mg; Zn, 110 mg; Mn, 40 mg; I, 1.3 mg; Se, 0.30 mg; Ethoxyquin, 150 mg.

to a scale based on average live weight. The daily feed supplied for pigs of the HO group increased from 1.0 to 3.3 kg. A 10% greater quantity of feed was supplied to the CH group in order to match the lower energy and protein content of CH feed and provide isoenergetic and isoproteic rations to the pigs of both groups. Fresh water was provided *ad libitum*. Ham subcutaneous fat samples were obtained *in vivo* by shot-biopsies at two different growth periods: growing

(44 kg mean BW and SD = 3 kg) and finishing (100 kg mean BW and SD=4.4 kg). In the first sampling biopsies were obtained before morning meal. In the last sampling, fat biopsies were obtained immediately before (animals fasted for 18h, from 16.00h of the previous day to 10.00h of sampling day) and three hours after feeding (postprandial sampling).

Pigs were tranquilized by intramuscular injection of 20 mg of azaperon per 10 kg live weight (Stresnil, Esteve, Barcelona, Spain) 1 h before micro-biopsies were taken. Biopsies were taken using a cylindrical biopsy device with a diameter of 5 mm under local anaesthesia with 2% lidocaine-HCL (Alphacaine, Fendigo). After biopsy, the zone was sprayed with oxytretracycline and lidocaine (Veterin Tenicol; Lab. Intervet S.A., Salamanca, Spain). Pigs did not suffer any pain because the employed analgesia. Similar procedures have been used before i.e. Barea *et al*. (2013). Biopsy samples were placed in cryotubes, snap frozen in liquid nitrogen and stored at -80ºC until analyses.

Candidate gene expression analyses by qPCR

The entire available adipose tissue sample (aprox. 50-100 mg) from each biopsy was used for total RNA extraction using RiboPureTM RNA isolation kit (Ambion, Austin, USA) following the manufacturer´s recommendations. RNA obtained was quantified using a NanoDrop equipment (NanoDrop Technologies, Wilmington, USA) and RNA quality was assessed with an Agilent bioanalyzer device (Agilent Technologies, Palo Alto, USA). The RNA Integrity Number (RIN) values obtained for all the samples were in the range 7.5 to 8.5. First-strand cDNA synthesis was carried out with Superscript II (Invitrogen, Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl containing 1 µg of total RNA and following the supplier's instructions.

***Selection of candidate genes*:** Ten candidate genes were selected. Peroxisome Proliferator Activated Receptor γ (*PPARG*) and Retinoic X receptor γ (*RXRG*) are involved in gene expression regulation of adipogenesis and lipogenesis processes (Kersten, 2001; Lefebvre *et al.*, 2010). Leptin (*LEP*) is a hormone produced by adipose tissue, which controls energy balance and has also local effects inhibiting lipogenesis in adipose tissue and promoting FA catabolism (Wang *et al*., 1999). Additionally, seven candidate genes with direct functional involvement in lipogenesis and FA transport were selected: *ME1*, *SCD*, *FASN*,

ACACA, *ELOVL5*, *ELOVL6* and *FABP5*. Also, *RXRG*, *LEP* and *ME1* genes were found to be DE conditional on dietary energy source in a previous transcriptome analysis at adipose tissue level (Óvilo *et al.*, 2014).

The expression of this panel of candidate genes was quantified by qPCR. Primer pairs used for quantification were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL sequences, covering different exons in order to assure the amplification of the cDNA. Sequence of primers and amplicon lengths are indicated in Supplementary Table S1. Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche, Basel, Switzerland). The qPCR reactions were prepared in a total volume of 20 µl containing 2.5 µl of cDNA (1/20 dilution), 10 µl of SYBR Green mix and 0.15 µM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95ºC for 10 min, followed by 45 cycles of 95ºC (15s) and 60ºC (1 min) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95ºC (15s) followed by 60ºC (20s) and ramp up to 95°C with acquired fluorescence during the ramp to 0.01°C/s. Data were analysed with LyghtCycler480 SW1.5 software (Roche, Basel, Switzerland). All points and samples were run in triplets as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. For each gene, PCR efficiency was estimated by standard curve calculation using four points of cDNA serial dilutions. Cycles to threshold (***Ct***) values were employed for the statistical analyses of differential expression. Data normalization was carried out using the most stable endogenous gene out of *GAPDH, ACTB, TBP* and *B2M*. Stability of endogenous genes was tested with genorm software (http://medgen.ugent.be/~jvdesomp/genorm/). *ACTB* gene was employed for normalization.

Statistical analyses of candidate gene expression data

Statistical analysis of gene expression data was carried out following the method proposed by Steibel *et al.* (2009) which consists in the analysis of *Ct* values for the target and endogenous genes simultaneously using a linear mixed model. The following model was used for this purpose in a joint analysis of all the recorded gene expression measures:

$$y_{gijkr} = TG_{gi} + P_{gj} + B_{gk} + D_{ijk} + e_{gijkr}$$

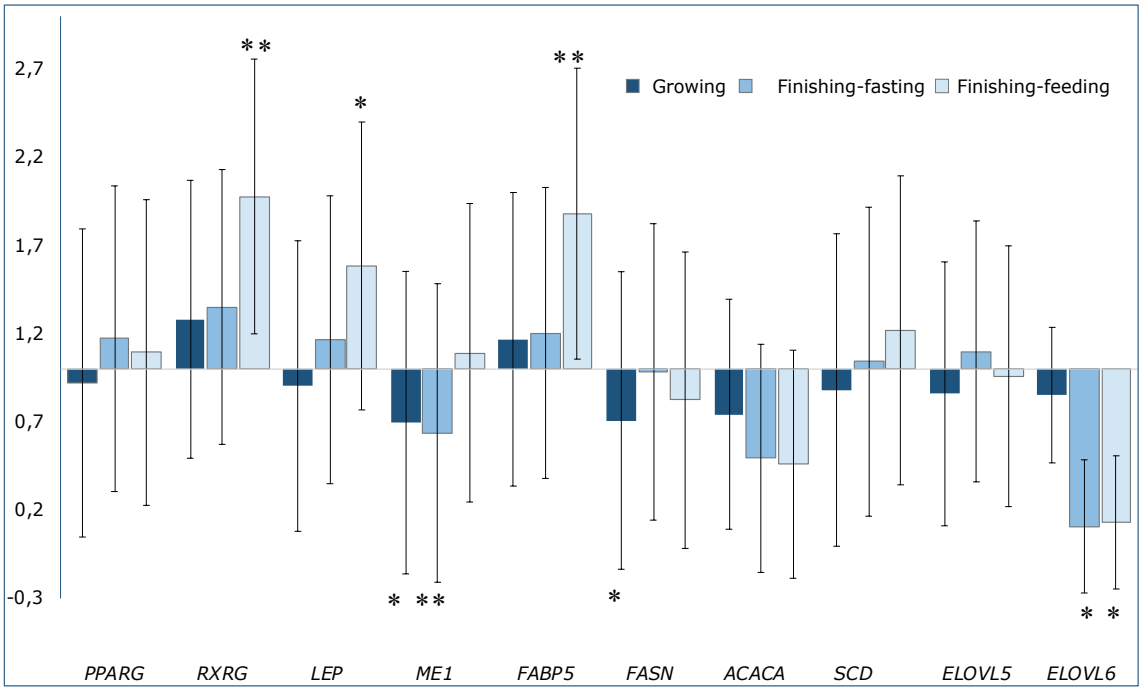
where $y_{gijkr} = -log_2\left(E_g^{-Ct_{gijkr}}\right)$, E_g brings the efficiency of the PCR of each gene, Ct_{gijkr} is the value obtained from the thermocycler software for the *g*th gene from the *r*th well in the *j*th qPCR plate corresponding to the the *k*th animal subjected to the *i*th treatment, *TGgi* is the specific effect of the *i*th treatment on the expression of gene *g*, P_{gj} and B_{gk} are specific random effects on the expression in the *j*th qPCR plate of gene *g* and the *k*th pig, D_{ik} is a random sample-specific effect common to all genes, and e_{gijkr} is a residual effect. Six different treatments were fitted to the model: dietary effects (two levels: HO and CH) in growing pigs (44 kg BW) and the four combinations of the two diets and fasting/feeding status in finishing pigs (100 kg BW).

To test differences or interaction between classes in the expression rate of genes of interest (***diff*$_{TG}$**) normalized by the endogenous gene, different contrasts were performed between the appropriate estimates of *TG* levels. Significance of $diff_{TG}$ estimates was determined with the *t* statistic. For accounting of the multiplicity of comparisons, adjusted *P*-values were calculated using the correction method of Benjamini and Hochberg (1995). These calculations were performed using the R Stats Package version 3.2.0 (https://stat.ethz.ch/R-manual/R-patched/ library/ stats/html/ p.adjust.html). To obtain fold change values (***FC***) from the estimated $diff_{TG}$ values, the following equation was applied: $FC = 2^{-diff_{TG}}$. Standard errors of fold change values (***SE (FC)***) were calculated from the standard error of the estimated differences (***SE***), using a similar transformation: $SE\ (FC) = 2^{-SE}$. Asymmetric confidence intervals (95% **CI**) were calculated for each *FC* value by using the *SE* values: 95% CI from $2^{-(diff_{TG}+t(\gamma,0.975).SE)}$ to $2^{-(diff_{TG}-t(\gamma,0.975).SE)}$, where $t(\gamma,0.975)$ is the 97.5 quantile of the Student-*t* distribution with γ degrees of freedom. In our analyses γ ranged from 57 to 103.

3.2.5. RESULTS

Diet effects on candidate gene expression in the three samplings are shown in Figure 1. In the first sampling (growing period, 44 kg average BW) obtained from fasted animals, diet influenced the expression of *ME1* and *FASN* genes, which showed higher expression in CH than in HO group (1.4x for both genes; $P<0.05$). Gene expression was also quantified in subcutaneous fat biopsies obtained before and three hours after feeding, in a sampling performed after a long period (23 weeks) of dietary treatment (finishing period, 100 kg of average BW). In the samples obtained before feeding, diet influenced the expression of *ME1* and *ELOVL6*, with both lipogenic genes showing also

Figure 1. Fold change (FC) ratios of candidate genes expression between Iberian pigs fed HO vs CH diets in ham subcutaneous adipose tissue biopsies obtained at growing, finishing-fasting and finishing-feeding. Error lines indicate the standard errors for the estimated FC values. FC values higher than 1 indicate higher expression in HO diet. *P<0.05 **P<0.01



higher expression in CH group (1.5x and 9x; P<0.01 and P<0.05, respectively). In the postpandrial sampling a higher number of statistically significant effects of diet was found. *ELOVL6* gene was upregulated in CH group (7.7x, P<0.05), while *RXRG*, *FABP5* and *LEP* were upregulated in HO group (1.98x, 1.88x, 1.58x, respectively; P<0.01 for *RXRG* and *FABP5* and P<0.05 for *LEP*).

Growth period significantly affected the expression of most of the analysed genes (Table 2). The genes *ACACA*, *FABP5*, *LEP*, *ME1*, *RXRG*, *FASN* and *SCD* showed higher expression in samples obtained at finishing (fold changes ranging from 1.4x for *FASN* gene to 88x for *LEP* gene); and *PPARG* showed higher expression in biopsies obtained in growing pigs (1.7x). No significant effect was found for the interaction diet*growth period. Feeding/fasting status effects on gene expression were also evaluated (Table 2). Only *PPARG* gene was significantly affected by feeding status, with postpandrial samples showing higher expression than fasting ones (1.9x, P<0.0001). This response was similar in both CH and HO groups. Regarding the interaction diet*feeding status, only one significant interaction effect was found for *ME1* gene (P<0.05). In finishing pigs, this gene was down-regulated by HO diet in pigs considered after an overnight fast, but expression

Table 2: Main effects of growth period, feeding/fasting status and diet on gene expression of candidate genes in ham adipose tissue of Iberian pigs.

Gene	Growth Period (Growing/Finishing)				Status (Fasting/Feeding)				Diet (HO[1]/CH[2])			
	Fold Change	95% CI[3]	*P*-value	Adjusted *P*- value	Fold Change	95% CI[3]	*P*-value	Adjusted *P*-value	Fold Change	95% CI[3]	*P*-value	Adjusted *P* value
ACACA	0.09	0.05-0.17	<.0001	0.001	0.89	0.48-1.65	0.710	0.919	0.55	0.34-0.91	0.021	0.104
ELOVL5	0.71	0.47-1.08	0.107	0.322	1.01	0.66-1.55	0.945	0.953	0.97	0.69-1.36	0.854	0.934
ELOVL6	3.71	0.95-14.43	0.059	0.219	1.12	0.28-4.41	0.871	0.934	0.23	0.08-0.70	0.010	0.060
FABP5	0.06	0.05-0.08	<.0001	0.001	0.94	0.72-1.24	0.657	0.889	1.38	1.11-1.72	0.004	0.036
FASN	0.71	0.55-0.91	0.008	0.050	1.05	0.82-1.34	0.701	0.917	0.83	0.68-1.01	0.071	0.255
LEP	0.01	0.01-0.02	<.0001	0.001	0.92	0.69-1.22	0.548	0.809	1.19	0.94-1.49	0.144	0.355
ME1	0.52	0.41-0.66	<.0001	0.001	1.03	0.82-1.30	0.791	0.932	-	-	-	-
PPARG	1.71	1.39-2.10	<.0001	0.001	0.52	0.43-0.64	<.0001	0.001	1.06	0.90-1.24	0.492	0.781
RXRG	0.03	0.02-0.05	<.0001	0.001	1.12	0.78-1.58	0.539	0.809	1.51	1.13-2.00	0.005	0.039
SCD	0.70	0.58-0.84	0.001	0.004	0.84	0.70-1.02	0.075	0.261	1.04	0.89-1.20	0.625	0.860

HO = high oleic diet with high oleic sunflower oil; CH = carbohydrate diet without added fat.
No significant growth period × diet interaction effect was found. A significant status × diet effect was found for *ME1* gene (P <0.05).

level did not differ between HO and CH diet when pigs were considered few hours after refeeding (Figure 1).

Taking into account the general lack of significant interactions among samplings, the diet effects on gene expression were better estimated from the whole sampling. These results are shown in Table 2 and indicate a higher expression of *ACACA* and *ELOVL6* in CH group; and a higher expression of *FABP5* and *RXRG* in HO group. For the *LEP* gene, which was significantly upregulated in HO group in the postprandial sampling, no significant diet effect was detected in the joint analysis of the three samplings. Due to the interaction effect, *ME1* gene expression differences due to diet are better estimated from the joint analysis of fasting samplings (1.5x in CH group, $P<0.001$).

Results with nominal *P* value <0.05 were considered statistically significant in the above paragraphs. Adjusted *P* values corrected for the multiplicity of performed tests were also reported in Table 2. This correction may be considered too demanding, due to the existent correlation between the expressions of many of the studied genes, which prevents to consider independent all the tests. In any case, all the nominally significant results showed adjusted *P*-values < 0.10, except the dietary effect on *FASN* gene expression (adjusted P-value < 0.17).

3.2.6. DISCUSSION

Our previous study (Óvilo *et al*., 2014) on the long term effects of HO/CH dietary intervention reported small magnitude and scarce differences in adipose tissue gene expression, suggesting a low genetic responsiveness of fat tissues to dietary treatments in the obese breed employed, at least in the long term. In this previous work, the effects of diet on tissue composition were observed to be established very early after the start of the treatment, suggesting that diet effects on metabolism could vary in response and intensity along the treatment. Our present results, obtained from the same animals employed in Óvilo *et al*. (2014), indicate that the growth period has an important effect on lipogenic gene expression, but the transcriptional response to the dietary intervention is only slightly modulated along time. Moreover, feeding status only marginally affected expression levels of targeted genes.

Candidate gene expression strongly differed between the two growth periods studied. Eight out of the ten analysed genes were significantly affected by growth

period, with most genes showing upregulation in the late sampling (finishing, 100 kg BW). Also, the magnitude of gene expression differences was strong, reaching 88-fold expression change for *LEP* gene. These results agree with those reported by Hood and Allen (1973) who found, in a fatter porcine line, a greater activity of lipogenic enzymes in older(and heavier) pigs. In this previous work a different pattern was found in leaner pig lines, in agreement with later findings in Meishan and Large-White pigs (Mourot *et al*., 1996). This ensemble of results may indicate differences in lipogenesis and its evolution along time between Iberian and both lean and other fat pig breeds. *PPARG* is the only gene showing higher expression in the early sampling (growing, 44 kg BW), in agreement with a more intense adipocyte differentiation which is expected in young animals.

Regarding the diet effect, it is interesting to note that the present results show a wider transcriptional response, involving more candidate genes, than the previous study. Nevertheless, these differences may be also related to the analysed samples: ham subcutaneous adipose tissue biopsies in the present work (including both layers) and backfat inner layer in the previous one; hindering the comparison. These results highlight the difficulty in the choice of tissue anatomical location in nutrigenomic or gene expression studies. In general, we observe an increase in the expression of lipogenic genes in CH group (*ME1*, *ELOVL6*, *ACACA*), which is in agreement with the expected greater *de novo* FA synthesis from the available dietary carbohydrates. Cytosolic malic enzyme (ME1) generates NADPH which contributes to *de novo* FA synthesis by FASN (Wise and Ball, 1964). ACACA and FASN are the two central enzymes of *de novo* lipogenesis, which use acetyl-CoA and malonyl-CoA derived from glucose or other carbon precursors to generate palmitate (Wakil *et al.*, 1983), which can be modified by endogenous elongase and desaturase enzymes to produce multiple lipid species. In particular the *ELOVL6* gene is a member of the elongation-of-very-long-chain-fatty-acid gene family of condensing enzymes that perform the first and rate-limiting step in the elongation cycle in mammals (Jakobsson *et al.*, 2006). The *ELOVL6* gene, which shows the diet-induced expression change of highest magnitude (9x), is involved in *de novo* lipogenesis, and catalyzes the elongation of long-chain SFA and MUFA. It is interesting to note that a different response to diet is observed for *ELOVL5* and *ELOVL6* genes, in spite of having a similar role. Their different behavior may be due to different substrate specificity and availability, as *ELOVL5*

preferentially elongates PUFA, while *ELOVL6* more effectively elongates SFA and MUFA (Jakobbsson *et al*., 2006; Green *et al*., 2010). The higher availability of SFA and MUFA synthetized from dietary CH, which is observed at the tissue composition level (Óvilo *et al*., 2014), would particularly induce the *ELOVL6* expression.

On the other hand, an increase in the expression of *RXRG* and *FABP5* genes is observed in HO group, mainly in finishing pigs, at postprandial state. Fatty acid binding proteins (**FABPs**) play an important role in the trafficking of free FAs and other lipids in different tissues (Zimmerman and Veerkamp, 2002), and thus, our results are in accordance with the higher dietary FA levels available for transport in HO pigs. Upregulation of *RXRG* in HO group was observed in the previous work analyzing backfat inner layer after a 24 weeks treatment (Óvilo *et al.*, 2014), and is in agreement with works reporting the activation of RXR receptors by different unsaturated fatty acids, including oleic acid (Lengqvist *et al*., 2004). RXRs are involved in adipogenesis, lipid and glucose homeostasis, and in particular, RXRG is involved in adipocyte survival, lipogenesis and hypertrophy (Lefebvre *et al.*, 2010). As previously proposed, the changes observed in the expression of *RXRG*, with higher expression in the HO group, could reflect a metabolic adaptation to a higher FA turnover. Differential expression results obtained for *RXRG* and *PPARG* genes are unexpected taking into account their synergistic roles. Both molecules are involved in the same adipogenic and lipogenic pathways, and work coordinately, acting as heterodimeric partners. A possible explanation may be related to the differential role of the several PPAR isoforms. *RXRG* response may be coordinated with functional changes in *PPARA* gene (not analysed in this work), which main role is related with FA catabolism and lipid storage, thereby protecting against lipotoxicity (Georgiadi and Kersten, 2012). On the other hand, PUFA are known ligands for *PPARG*, but a regulatory role by MUFA is not known for this gene, contrarily to *RXRG* evidences.

The Stearoyl-CoA desaturase (*SCD*) gene encodes an endoplasmic reticulum enzyme that catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids (Paton and Ntambi, 2009). Previous results observed *in vitro* and *in vivo* in other pig breeds and species, showed that oleic acid inhibits *SCD* expression, while high carbohydrate diets induces its expression (Zulfikli *et al*., 2010; Duran-Montgé *et al*., 2009; Paton and Ntambi, 2009). Nevertheless, in the present study no change on *SCD* gene expression is observed conditional on diet HO/CH content. Moreover, FA composition data available from the growing biopsies

(Óvilo *et al.*, 2014) indicate that the different diet composition results in a significant difference in the oleic acid content in adipose tissue (47.36 ± 0.37 *vs* 56.78 ± 0.36 in CH and HO groups, $P<0.001$). These results suggest a lack of an inhibitory effect of oleic acid on *SCD* gene expression in Iberian pigs, although regulation of the SCD at protein expression or activity levels cannot be discarded (Doran *et al*., 2006).

At last, we have to take into account that, in order to provide isocaloric regimens, animals receiving HO diet also had a higher fiber content in the diet. Dietary fiber is known to influence nutrient utilization and feed intake and interacts with gut micro-environment. In our experiment there were no differences in feed intake as pigs ate the complete provided rations (no feed refusals were recorded), but differences in nutrient utilization and fat metabolism due to different fiber levels cannot be discarded.

Fasting, including short-term food deprivation, is known to reduce lipogenesis and induce lipolysis (Kersten *et al*., 2001; Palou *et al*., 2010). Nevertheless, in our work feeding/fasting status only affected the expression of *PPARG* gene, which is upregulated after feeding. In agreement, previous data have shown down-regulation of *PPARG* with fasting, but in that situation, other lipogenic regulators were also repressed (Houseknecht *et al*., 1998; Morgan *et al.*, 2008). The lack of effects of fasting on lipogenic enzymes expression observed in the present study is unexpected and could be related to the duration of the fasting applied. Probably, after short or medium fasting periods lipogenesis regulators are affected, while longer fasting periods may lead to a higher response in downstream genes. In fact, acute fasting (72h) has been reported to produce greater effect on *LEP* than on *PPARG* genes expression (O'Gorman *et al*., 2010). On the other hand, results could be consistent with findings indicating that, in obese animals, *de novo* lipogenesis persist in liver and adipose tissue during fasting (Morgan *et al.*, 2008). Persistence of lipogenesis during fasting, with independence of the diet, may be a characteristic of the Iberian pigs employed. In other words, a longer food deprivation period than the one applied (standard overnight fasting) could be necessary to stop lipogenesis and induce lipolysis in these fatty animals.

Also, no differences in *SCD* gene expression are observed between the two physiological states compared in the present work (fasting *vs* feeding), indicating a stable *SCD* gene expression independent of nutritional factors. This is in agreement with the high desaturation potential and the high capacity of the breed

to storage high quantities of oleic acid in their tissues, narrowly related with its sensorial and nutritional meat quality (López-Bote, 1998; Barea *et al*., 2013).

In conclusion, both in the short/medium term and in the long term, several transcriptional adaptations are observed in ham subcutaneous adipose tissue following a nutritional intervention with MUFA/CH enriched diets. Results regarding diet effects are coherent in the very different timings analysed, in spite of strong differences in candidate gene expression between growth periods, and compatible with a higher *de novo* endogenous synthesis of FA in the carbohydrate-supplemented group and a higher FA transport in the oleic acid-supplemented group. Results from the comparison of feeding and fasting samples suggests persistence of *de novo* lipogenesis during fasting, which may be a characteristic trait of the fatty phenotype of the employed Iberian breed.

Acknowledgments

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Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S1751731115003055

3. CAPITULO 3

Efectos moduladores de la raza, el estado de alimentación y la dieta sobre la expresión de genes adipogénicos, lipogénicos y lipolíticos en cerdos ibéricos y duroc en crecimiento

Modulatory effects of breed, feeding status and diet on adipogenic, lipogenic and lipolytic gene expression in growing Iberian and Duroc pigs.

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Modulatory Effects of Breed, Feeding Status, and Diet on Adipogenic, Lipogenic, and Lipolytic Gene Expression in Growing Iberian and Duroc Pigs

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Abstract: Meat quality depends on tissue composition which is in turn influenced by different factors, such as diet, genotype, age, or sex. We evaluated the effects of breed, 24 h fasting, and dietary energy source (HO: oleic acid versus CH: carbohydrates) on the expression of candidate genes involved in adipogenesis, lipogenesis, and lipolysis in the adipose tissue from Iberian and Duroc growing pigs. The Iberian pigs showed greater feed intake, backfat thickness, and saturated fatty acids (SFA) content in the subcutaneous fat, whereas the Duroc pigs had greater ham weight and polyunsaturated fatty acids (PUFA) content. In both breeds, the diet induced changes in the fatty acid (FA) composition of subcutaneous fat samples. The HO group had higher monounsaturated fatty acids (MUFA) and oleic acid, and lower SFA than the CH group. Regarding gene expression, breed and feeding status (fasting versus postprandial) had significant effects on gene expression, with quantitative interactions between them, while diet showed negligible effects. In general, adipogenic and lipogenic genes were upregulated in the Iberian pigs and in postprandial samples. In contrast, the expression of lipolytic genes showed complex interaction effects. Our results agree with the phenotypic differences between the Iberian and Duroc breeds and with the inhibition of lipogenesis by fasting. Quantitative interactions between breed and feeding status effects were observed, which indicates a different response to fasting of the two breeds, with the obese Iberian breed showing a more stable expression of lipogenic genes. These results highlight the complexity of lipid metabolism regulation, especially in relation to lipolysis processes.

Keywords: nutrigenomics; diet; fasting; adipose tissue; lipid metabolism; gene expression and Iberian pig

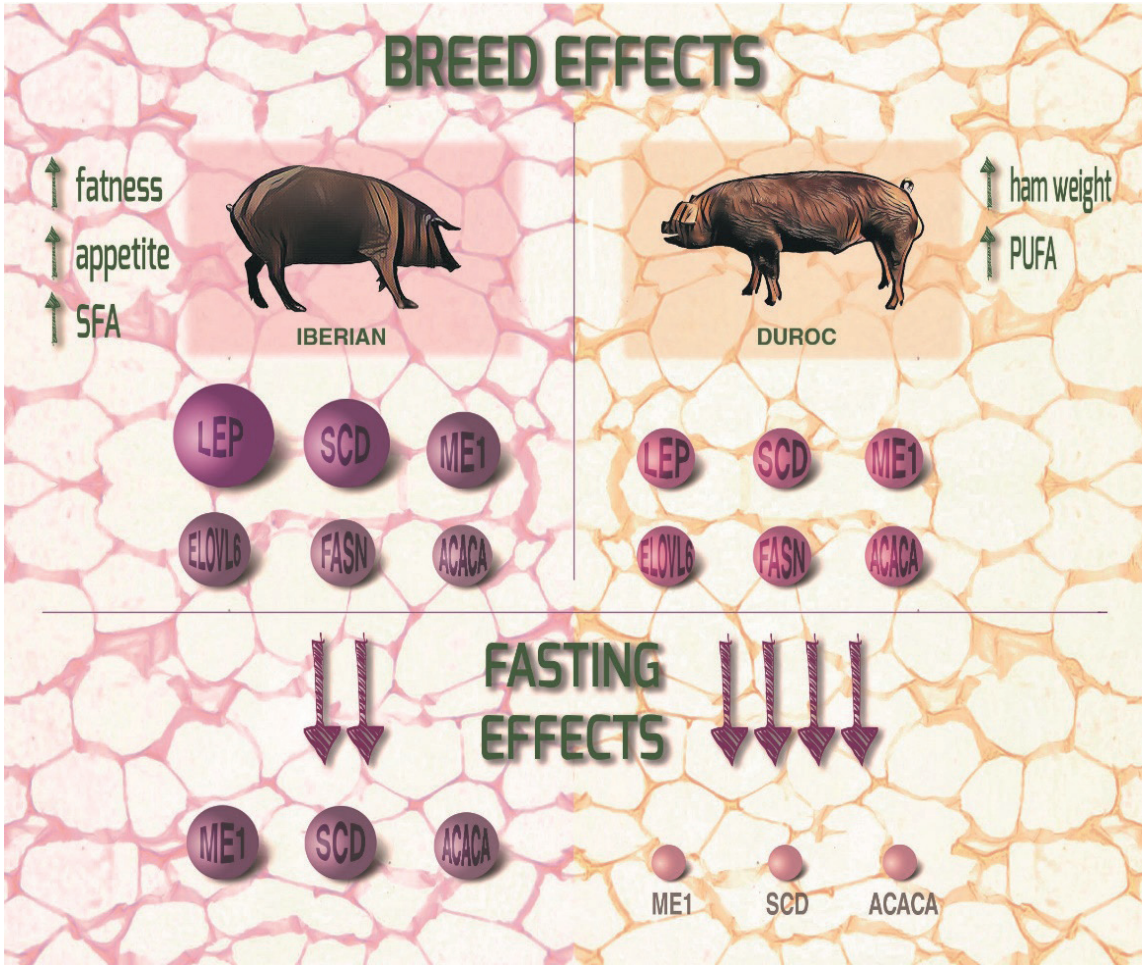
1. Introduction

The Iberian pig is the main representative of the Mediterranean traditional fatty pig breeds. This breed has an outstanding commercial and socioeconomic value because of the employment of natural resources in its extensive production system and because it is the basis for the production of high quality dry-cured meat products [1]. The Iberian breed is characterized by a great appetite, fatness, and protein turnover ratio resulting in low lean growth efficiency [2], and is traditionally fed according to a feeding system based on the intake of acorns and on pasture, in the final fattening period. This fattening system is a reference model for the sustainable production of many local Mediterranean

3.3.1. ABSTRACT

Meat quality depends on tissue composition which is in turn influenced by different factors as diet, genotype, age or sex. We evaluated the effects of breed, 24 h-fasting and dietary energy source (HO: oleic acid vs CH: carbohydrates) on expression of candidate genes involved in adipogenesis, lipogenesis and lipolysis in adipose tissue from Iberian and Duroc growing pigs. Iberian pigs showed greater feed intake, backfat thickness and saturated fatty acids (SFA) content of subcutaneous fat, whereas Duroc pigs had a greater ham weight and polyunsaturated fatty acids (PUFA) content. In both breeds, the diet induced changes in fatty acid (FA) composition of subcutaneous fat samples. HO group had higher monounsaturated fatty acids (MUFA) and oleic acid and lower SFA than CH Regarding gene expression, breed and feeding status (fasting vs postprandial) had significant effects on gene expression, with quantitative interactions between them, while diet showed negligible effects. In general, adipogenic and

Graphical Abstract



lipogenic genes were upregulated in Iberian pigs and in postprandial samples. In contrast, lipolytic genes expression showed complex interaction effects. Results agree with the phenotypic differences between Iberian and Duroc breeds and with the inhibition of lipogenesis by fasting. Breed x feeding status quantitative interactions were observed, which indicate a different response between breeds to fasting, with the obese Iberian breed showing a more stable expression of lipogenic genes. Results highlight the complexity of lipid metabolism regulation especially in relation to lipolysis processes.

Keywords: nutrigenomics; diet; fasting; adipose tissue; lipid metabolism; gene expression and Iberian pig.

3.3.2. INTRODUCTION

The Iberian pig is the main representative of the Mediterranean traditional fatty pig breeds. This breed has an outstanding commercial and socio-economic relevance because of the employment of natural resources in its traditional extensive production system and because it is the basis for the production of high quality dry-cured meat products (López-Bote, 1998). The Iberian breed is characterized by a great appetite, fatness and protein turnover ratio resulting in low lean growth and efficiency (Ayuso *et al*., 2016) and traditional feeding system with final fattening based on the intake of acorns and pasture. This fattening system is a reference model for sustainable production of many local Mediterranean breeds (Pugliese *et al*., 2012). Iberian pig production is based on both purebred Iberian and crossbred Duroc x Iberian pigs.

These two breeds show important phenotypic differences in growth, fattening, tissue composition, muscle differentiation and several metabolic processes from early developmental stages (Ayuso *et al*., 2016; Pérez-Enciso *et al*., 2009). Specifically, purebred Iberian animals are characterized by a lower lean growth efficiency and higher meat quality than Duroc genotypes, although a precise characterization of the phenotypic differences between both pure breeds has not been reported yet.

Fat content and composition largely determine the meat quality (Wood *et al*., 2008), and are influenced by individual factors, including genetic background, age and sex, and also by environmental factors such as nutritional composition. Diet is a main factor influencing animal body and tissue composition, and may be employed to improve the nutritional value of meat (Raes *et al*., 2004). Diet

components can be directly stored in the animal tissues and they may also affect metabolism, by influencing gene expression. MUFA enriched diets, through the inclusion of high oleic acid sunflower oil, are being used in Iberian pig intensive fattening systems in order to increase mainly oleic acid and to be used as an alternative to traditional fattening in Montanera (Ventanas *et al*., 2008; Pérez-Palacios *et al*., 2004). Recently, the impact of high oleic sunflower oil (HO) *vs* carbohydrate (CH) enriched diets on tissue composition and their short- and long-term effects on gene transcription have been evaluated in pure Iberian pigs (Óvilo *et al*., 2004a; Martins *et al*., 2015; González *et al*., 2012). In previous works, the effects of diet composition on gene expression were small and conditioned by several factors such as timing and feeding status. Moreover, diet effects on metabolism may depend on the peculiar breed/genotype employed. Specifically, the high basal lipogenesis characteristic of Iberian breed tissues may be associated to a lower responsiveness to dietary interventions regarding lipid metabolism.

Also, in our previous work performed with Iberian pigs, (Benítez *et al*., 2016) the effects of a period of 18 h-fasting on lipogenic gene expression was tested. This medium-term fasting only downregulated the expression of the PPARG gene among a panel of lipogenesis candidates. The scarce transcriptional response to fasting was unexpected and could be related to the duration of the fasting applied, as longer fasting periods are expected to induce a higher response in gene expression. On the other hand, these scarce effects of fasting could be also conditional on the genetic type employed (pure Iberian animals) in which the high lipogenesis potential may limit or delay the transcriptional response to the lack of energy supply. Understanding of the molecular mechanisms driving the high potential for fat deposition in Iberian pigs in comparison to other breeds, even in adverse situations of caloric restriction, would have a relevant scientific interest from an animal genetics and production perspective. Moreover, it also could provide relevant data to deepen the knowledge of these complex processes in other obese animals including humans, as the Iberian pig is considered an adequate animal model for obesity and metabolic alterations (Torres-Rovira *et al*., 2012).

Fat deposition is the result of the balance between lipogenesis and lipolysis processes (Pena *et al*., 2014). Previous nutrigenomic works addressed the study of lipogenesis at the transcriptional level, following diet and fasting treatments (Óvilo *et al*., 2014a; Benítez *et al*., 2016) On the other hand, previous transcriptome

studies have compared muscle gene expression of Iberian vs crossbred animals (Óvilo et al., 2014a; Óvilo et al., 2014b). These studies suggested a more active lipogenesis in pure Iberian animals, but also a few lipolytic genes were differentially expressed between genotypes, concluding the relative contribution of both processes in the final fat deposition differences (Óvilo et al., 2014b) and highlighting the interest of studying both processes simultaneously.

Thus, in order to better understand the diet and fasting effects on gene transcription and lipid metabolism and the potential between-breeds differential response, Iberian and another reference lean breeds must be jointly studied in the same experimental conditions and using both a dietary intervention and a longer fasting period. In agreement with previous considerations, this experiment was conducted to investigate breed (Iberian vs Duroc), a long period of fasting (24 h) and dietary energy source (HO vs CH) effects on adipose tissue composition and gene expression of candidate genes involved in adipogenesis, lipogenesis and lipolysis in growing pigs.

3.3.3. RESULTS

Growth, fattening and FA composition data corresponding to the pig samples obtained at slaughter were employed to analyze the breed and diet effects. Adipose tissue samples obtained by biopsy four days before slaughter were employed to study the effects of breed, diet and 24 h fasting on gene expression.

3.3.3.1. Diet and breed effects on phenotype and animal tissue composition

The two dietary groups (HO, CH) showed similar weight, backfat thickness and ham weight at the end of the experiment. Regarding breed effects, higher backfat thickness (24.1 mm vs 10.7 mm in loin and 27.8 mm vs 15.7 mm in ham, $P< 0.001$), higher average feed intake (2 kg vs 1.7 kg, $P< 0.05$) were registered in Iberian pigs and higher ham weight (4.5 kg vs 3.5 kg, $P< 0.001$) was registered in Duroc pigs.

The FA profile was studied in two different tissues: subcutaneous backfat and subcutaneous ham fat (inner and outer layers). Behavior of both layers was very similar at each location, and thus results of diet and breed effects are presented

Table 1. Least-squares means and standard errors (SEM) of fatty acid percentages in subcutaneous backfat (inner layer) from Iberian and Duroc pigs fed with a diet enriched with high-oleic sunflower oil (HO) or a standard diet with carbohydrates as energy source (CH)

Fatty acid[1]	Diet CH		Diet HO			adjusted	DUROC		IBERIAN			adjusted
	MEAN	SEM	MEAN	SEM	*P*-value	*P*-value	Mean	SEM	Mean	SEM	*P*-value	*P*-value
C14:0	1.21	0.03	1.12	0.03	0.05	ns	1.08	0.03	1.25	0.03	0.005	ns
C16:0	23.68	0.23	20.91	0.23	<.0001	0.0007	20.32	0.27	24.27	0.20	<.0001	0.0007
C16:1n-9	0.33	0.01	0.44	0.01	<.0001	0.0007	0.45	0.02	0.31	0.01	<.0001	0.0007
C16:1n-7	1.97	0.09	1.60	0.08	0.009	ns	1.97	0.08	1.60	0.10	0.01	ns
C17:0	0.46	0.03	0.39	0.03	0.14	ns	0.41	0.04	0.44	0.03	0.60	ns
C17:1	0.35	0.02	0.28	0.02	0.001	0.04	0.29	0.02	0.34	0.02	0.15	ns
C18:0	13.35	0.24	10.35	0.22	<.0001	0.0007	10.31	0.26	13.39	0.21	<.0001	0.0007
C18:1n-9	40.23	0.53	46.49	0.50	<.0001	0.0007	44.20	0.61	42.53	0.46	0.06	ns
C18:1n-7	1.64	0.13	1.68	0.12	0.78	ns	1.77	0.15	1.55	0.12	0.30	ns
C18:2n-6	13.42	0.22	13.41	0.20	0.99	ns	16.13	0.25	10.70	0.20	<.0001	0.0007
C18:3n-3	0.84	0.01	0.88	0.01	0.02	ns	1.00	0.02	0.72	0.01	<.0001	0.0007
C18:4n-3	0.06	0.00	0.07	0.00	0.0004	0.03	0.07	0.00	0.06	0.00	0.01	ns
C20:0	0.22	0.01	0.18	0.01	0.002	ns	0.18	0.01	0.22	0.01	0.01	ns
C20:1n-9	0.97	0.03	1.03	0.02	0.03	ns	0.90	0.03	1.10	0.03	0.0002	0.01
C20:2	0.68	0.01	0.61	0.01	0.004	ns	0.71	0.02	0.59	0.01	0.0002	0.01
C20:4n-6	0.22	0.01	0.22	0.01	0.72	ns	0.26	0.01	0.18	0.01	<.0001	0.0007
C20:3n-3	0.12	0.00	0.11	0.00	0.02	ns	0.12	0.00	0.11	0.00	0.01	ns
C22:4n-6	0.10	0.00	0.09	0.00	0.27	ns	0.10	0.01	0.09	0.00	0.25	ns
C22:5n-3	0.09	0.02	0.09	0.01	0.69	ns	0.07	0.02	0.11	0.01	0.11	ns
C22:6n-3	0.03	0.01	0.05	0.01	0.20	ns	0.04	0.01	0.04	0.01	0.98	ns
SFA[2]	38.93	0.55	32.96	0.51	<.0001	0.0007	32.31	0.61	39.57	0.47	<.0001	0.0007
MUFA[3]	45.13	0.79	51.24	0.73	<.0001	0.0007	49.28	0.89	47.09	0.72	<.0001	0.0007
PUFA[4]	14.86	0.29	14.93	0.26	0.34	ns	17.79	0.32	12.01	0.26	<.0001	0.0007
n-6/n-3	12.05	0.01	11.43	0.01	0.04	ns	12.78	0.01	11.19	0.01	<.0001	0.0007

[1]Fatty acid composition is expressed as percentage (wt/wt) of total fatty acids.2SFA, 3MUFA, 4 PUFA = sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively.

Table 2. Least-squares means and standard errors (SEM) of fatty acid percentages in subcutaneous ham fat (inner layer) from Iberian and Duroc pigs fed with a diet enriched with high-oleic sunflower oil (HO) or a standard diet with carbohydrates as energy source (CH).

Fatty acid[1]	Diet CH		Diet HO			adjusted	DUROC		IBERIAN			adjusted
	MEAN	SEM	MEAN	SEM	*P*-value	*P*-value	MEAN	SEM	MEAN	SEM	*P*-value	*P*-value
C14:0	1.24	0.02	1.18	0.02	0.02	ns	1.15	0.02	1.27	0.02	0.0008	0.05
C16:0	22.57	0.22	20.54	0.20	<.0001	0.0007	20.38	0.23	22.73	0.18	<.0001	0.0007
C16:1n-9	0.28	0.01	0.39	0.01	<.0001	0.0007	0.36	0.02	0.31	0.01	0.02	ns
C16:1n-7	2.57	0.08	2.16	0.07	<.0001	0.0007	2.28	0.09	2.45	0.07	0.17	ns
C17:0	0.33	0.03	0.41	0.03	0.17	ns	0.33	0.04	0.42	0.03	0.13	ns
C17:1	0.40	0.02	0.31	0.02	0.0009	0.06	0.30	0.03	0.41	0.02	0.0099	ns
C18:0	10.48	0.21	8.79	0.19	<.0001	0.0007	9.33	0.23	9.94	0.18	0.05	ns
C18:1n-9	44.75	0.51	49.40	0.48	<.0001	0.0007	47.26	0.57	46.89	0.43	0.64	ns
C18:1n-7	1.84	0.12	1.69	0.11	0.27	ns	1.67	0.13	1.85	0.10	0.34	ns
C18:2n-6	12.11	0.19	11.87	0.17	0.53	ns	13.58	0.21	10.41	0.16	<.0001	0.0007
C18:3n-3	0.80	0.02	0.80	0.01	0.45	ns	0.87	0.02	0.73	0.02	<.0001	0.0007
C18:4n-3	0.08	0.00	0.09	0.00	0.0016	ns	0.09	0.00	0.08	0.00	0.26	ns
C20:0	0.16	0.01	0.14	0.00	<.0001	0.0007	0.15	0.01	0.15	0.01	0.50	ns
C20:1n-9	1.00	0.02	1.04	0.02	0.05	ns	0.91	0.02	1.13	0.02	<.0001	0.0007
C20:2	0.67	0.01	0.61	0.01	0.0004	0.03	0.68	0.01	0.61	0.01	<.0001	0.0007
C20:4n-6	0.25	0.01	0.25	0.01	0.56	ns	0.27	0.01	0.23	0.01	0.04	ns
C20:3n-3	0.13	0.00	0.13	0.00	0.03	ns	0.13	0.00	0.11	0.00	0.02	ns
C22:4n-6	0.10	0.00	0.09	0.00	0.05	ns	0.10	0.00	0.10	0.00	0.46	ns
C22:5n-3	0.13	0.01	0.11	0.01	0.30	ns	0.09	0.02	0.15	0.01	0.03	ns
C22:6n-3	0.07	0.01	0.07	0.01	0.43	ns	0.07	0.01	0.07	0.01	0.78	ns
SFA[2]	34.78	0.48	31.06	0.44	<.0001	0.0007	31.33	0.52	34.52	0.42	<.0001	0.0007
MUFA[3]	50.43	0.75	54.68	0.70	<.0001	0.0007	52.48	0.83	52.63	0.64	0.99	ns
PUFA[4]	13.67	0.25	13.41	0.23	0.34	ns	15.20	0.28	11.87	0.22	<.0001	0.0007
n-6/n-3	11.56	0.01	10.19	0.01	0.04	ns	11.16	0.01	9.42	0.01	<.0001	0.0007

[1]Fatty acid composition is expressed as percentage (wt/wt) of total fatty acids.2SFA, 3MUFA, 4PUFA= sum of saturated, monounsaturated and polyunsaturated fatty acids, respectively.

Table 3. Main effects of breed (Iberian /Duroc), feeding/fasting status and diet (HO/CH) on expression of candidate genes in ham adipose tissue.

Gene	Breed (Iberian/Duroc) (n = 30/19)				Status (Feeding/Fasting) (n = 48/48)				Diet (HO[1]/CH[2]) (n = 27/22)			
	Fold Change	95% CI[3]	*P*-value	Adjusted *P*- value	Fold Change	95% CI[3]	*P*-value	Adjusted *P*-value	Fold Change	95% CI[3]	*P*-value	Adjusted *P* value
RXRG	1.33	0.90-1.96	0.1424	0.196	1.27	1.08-1.49	0.0051	0.003	1.12	0.91-1.39	0.2720	0.913
PPARG	1.07	0.84-1.36	0.5434	0.213	1.22	1.07-1.38	0.0029	0.011	0.87	0.69-1.09	0.168	0.328
SREBP1	0.87	0.65-1.17	0.3153	0.434	1.11	0.93-1.32	0.234	0.322	0.99	0.74-1.33	0.964	0.970
LEP	2.85	1.23-6.64	0.0174	0.075	2.05	1.69-2.48	<.0001	0.001	0.99	0.68-1.45	0.970	0.970
ME1	1.80	1.03-3.14	0.0427	0.024	1.12	1.00-1.24	0.0470	0.117	1.18	0.70-2.00	0.4912	0.201
SCD	2.21	1.49-3.27	0.0009	0.003	1.30	1.21-1.40	<.0001	0.001	1.05	0.74-1.49	0.7304	0.893
ACACA	1.49	0.90-2.46	0.1068	0.147	2.10	1.87-2.35	<.0001	0.001	0.97	0.61-1.53	0.8709	0.871
FASN	1.36	0.95-1.94	0.0820	0.113	1.13	1.02-1.24	0.0196	0.036	1.16	0.81-1.63	0.3655	0.804
ELOVL6	1.60	1.06-2.40	0.0280	0.039	1.22	1.10-1.37	0.0005	0.001	0.94	0.68-1.31	0.664	0.809
ATGL	0.89	0.78-1.01	0.0640	0.088	0.99	0.92-1.07	0.811	0.811	1.01	0.92-1.11	0.799	0.879
HSL	0.94	0.79-1.13	0.4870	0.670	0.96	0.89-1.03	0.275	0.336	0.96	0.82-1.14	0.640	0.805
PLIN1	1.93	0.90-4.14	0.0873	0.120	1.07	0.92-1.25	0.399	0.439	0.79	0.63-1.00	0.050	0.046
G0S2	0.89	0.47-1.70	0.6887	0.814	1.50	1.16-1.95	0.003	0.007	0.82	0.51-1.30	0.321	0.501

[1]HO = high oleic diet with high oleic sunflower oil; [2]CH = carbohydrate diet without added fat; [3]CI: Confidence Interval

and discussed for the inner layer (Tables 1 and 2). In both locations (backfat and ham fat) FA composition showed significant differences between dietary groups and breeds. Main diet effects were observed for SFA and MUFA contents. The HO group showed higher MUFA and the CH group showed higher SFA content. These differences were mainly due to higher palmitic (C16:0) and stearic (C18:0) acids in the CH and higher palmitoleic (C16:1) and oleic (C18:1) in the HO group. The n-6/n-3 ratio was slightly higher in the CH diet in both locations.

In backfat, main breed effects were observed for SFA, MUFA and PUFA contents, with higher SFA in Iberian pigs and higher MUFA and PUFA in Duroc pigs. These changes were mainly due to a major increase of palmitic and stearic acids in Iberian pigs and an increase of palmitoleic, linoleic, linolenic and eicosadienoic fatty acids in Duroc pigs; although some other minority FA were also affected. No significant difference in oleic acid content was observed between breeds.

Similarly, in ham fat, main breed effects were observed for SFA and PUFA contents, with higher SFA in Iberian pigs and higher PUFA in Duroc pigs. These effects were a consequence of higher palmitic acid in Iberian and higher linoleic and linolenic acids in Duroc. The n-6/n-3 ratio was higher in Duroc pigs in both adipose depots. This change was in agreement with higher levels of linoleic acid in Duroc pigs, which is the main representative of n-6 FA and the precursor of the synthesis of arachidonic acid (C20:4n-6).

Only two significant diet x breed interactions were observed in subcutaneous backfat for eicosadienoic (C20:2) and eicosatrienoic (C20:3n-3) fatty acids with a higher amount of these FA in Duroc pigs fed CH diet than in the rest of the groups.

3.3.3.2. Breed, feeding status and diet effects on candidate gene expression

The thirteen selected candidate genes were successfully quantified in mRNA samples from ham subcutaneous adipose biopsies obtained in vivo by short-biopsies after a 24 h-fasting and 3 h after refeeding.

Results of breed, feeding status and diet effects on gene expression are shown in **Table 3** and **Figure 1.** Breed significantly affected the expression of several analyzed genes. *LEP, ME1, SCD*, and *ELOVL6* showed higher expression in biopsies obtained from Iberian pigs and *FASN* and *PLIN1* showed the same trend.

Figure 1. Fold change ratios (FC) of candidate genes expression in ham subcutaneous adipose biopsies (A) Iberian (n=29) vs Duroc (n=19) (B) Feeding vs fasting status (n=48). Error lines indicate the standard errors. FC values >1 indicate higher expression in Iberian pigs and feeding status. **P*<0.05,***P*<0.001, ****P*<0.0001

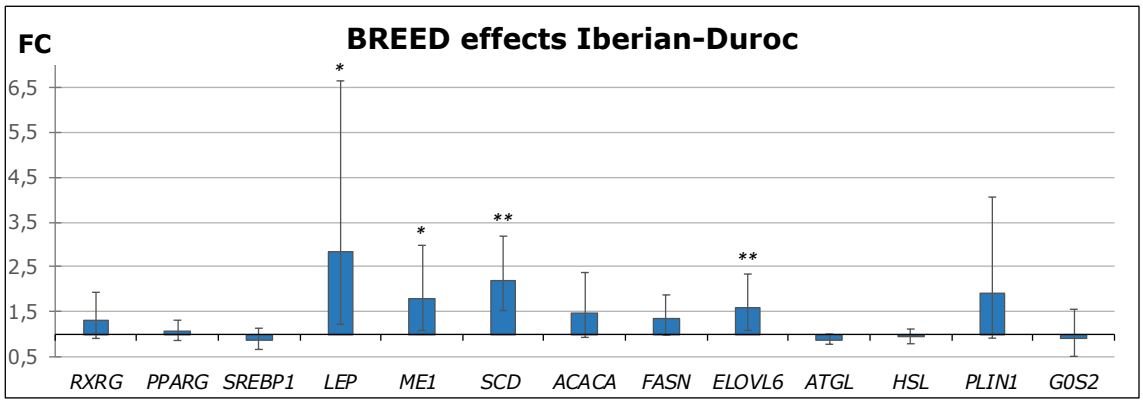


Figure 1A

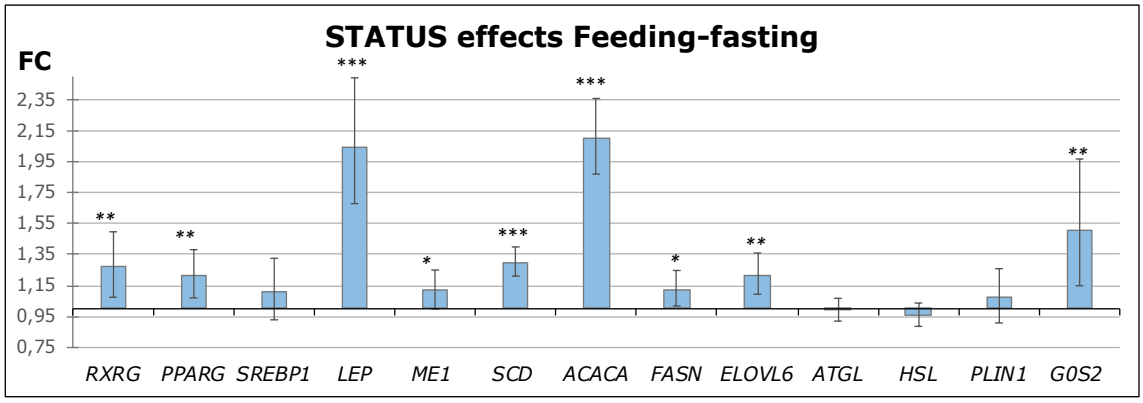


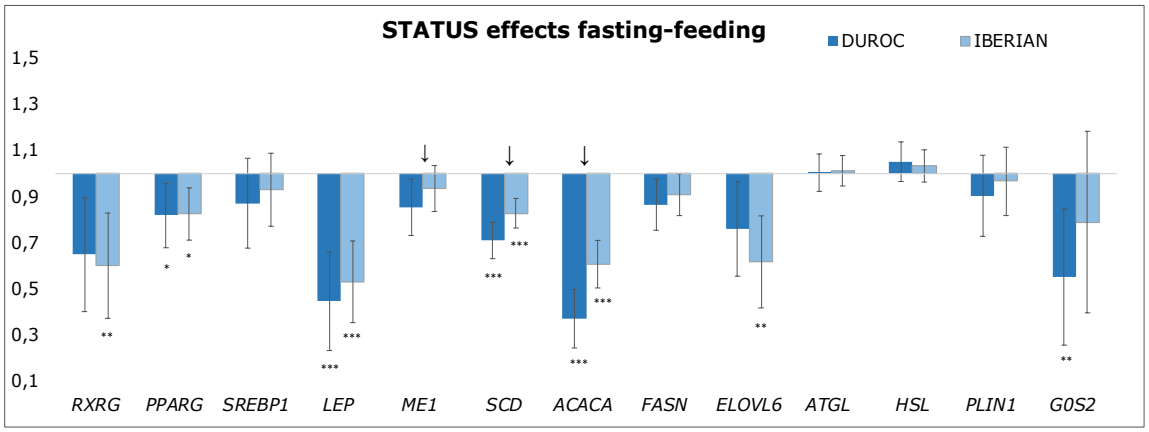
Figure 1B

On the contrary, the expression of the lipolytic gene *ATGL* tended to be higher in Duroc samples. Fold change (FC) ratios of *SCD* and *LEP* genes were considerably high for the breed effects **(Figure 1A).** FC values ranged from 2.85x for the LEP gene to 1.60x for the *ELOVL6* gene.

Fasting/feeding status significantly affected the expression of most analyzed genes. *RXRG, PPARG, LEP, ME1, SCD, ACACA, FASN, ELOVL6* and *G0S2* showed higher expression in biopsies obtained in postprandial status. *SREBP1, ATGL, HSL* and *PLIN1* were not affected by feeding status. FC ratios calculated for *ACACA* and *LEP* genes were considerably high for the status effect (Figure 1B). FCs ranged from 2.10x for the *ACACA* gene to 1.12x for the *ME1* gene.

Diet showed negligible effects on gene expression. Only the *PLIN1* gene was upregulated in the CH group. The results corresponding to the interaction

Figure 2. Fold change (FC) ratios of candidate genes expression between fasting vs feeding status in ham subcutaneous adipose biopsies obtained from Duroc (n=19) and Iberian (n=29) pigs. Error lines indicate the standard errors. FC values >1 indicate higher expression in fasting status. *P<0.05, **P<0.01,***P<0.0001. Breed*status interaction effects are indicated with an arrow (↓) (P<0.07, P<0.03 and P<.0001 for *ME1, SCD* and *ACACA* respectively)



effects are shown in **Table 4** and in **Figures 2, (Figure S1 and S2.)** Significant breed*status interaction effects were found for the *SCD* and *ACACA* genes (P<0.03 and P>0.0001, respectively) and a tendency for the *ME1* (P<0.07) **(Figure 2).**

A significant breed*diet interaction effect was found for the *ATGL* gene (P<0.006), in addition, significant diet*status interaction effects were found for *SREBP1, ACACA, ATGL, HSL* and *PLIN1* (P<0.03, P<0.004, P<0.02, P<0.03 and P<0.0004 respectively) **(Table 4 and Figures S1 and S2).**

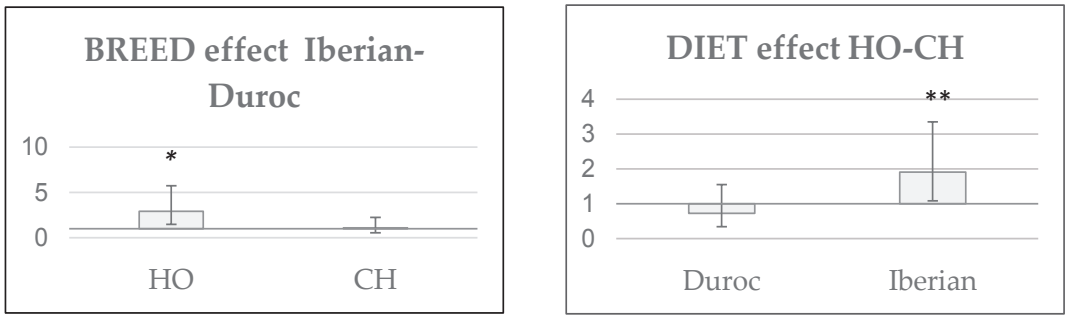
3.3.4. DISCUSSION

3.3.4.1. Diet and breed effects on phenotype and tissue composition

The effects of dietary energy source (monounsaturated oil or carbohydrate) on phenotype are in agreement with previous studies. Different works have reported no effect of dietary fat source or saturation on growth performance and carcass characteristics in pigs (Kouba *et al*., 2003; Morel *et al*., 2006; Olivares *et al*., 2010), matching our findings. In relation to FA composition, our study shows significant effects of diet in both breeds and in both subcutaneous backfat and ham fat fatty acid profiles. Both depots respond in a similar way to the diet reflecting in part the composition of the diet received, with much higher MUFA in the HO group, due to a major increase in oleic acid, as expected. The content of SFA was higher in the CH group, although CH diet included a lower proportion of

Figure S1. Breed*diet interactions

ME1

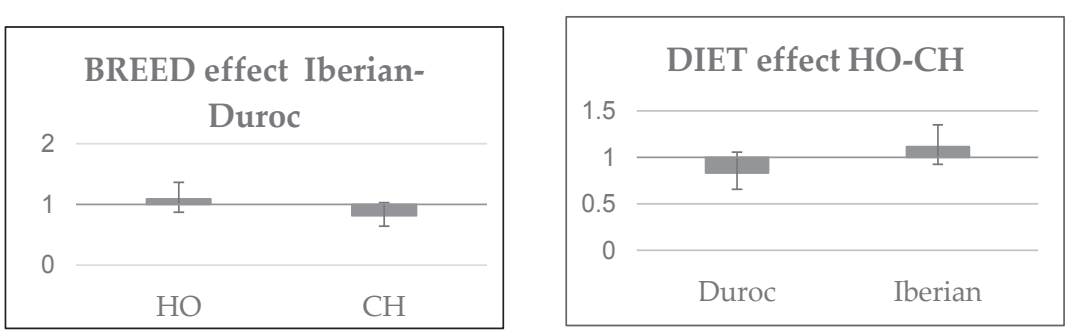


Breed*diet interaction *P*<0.07

ATGL

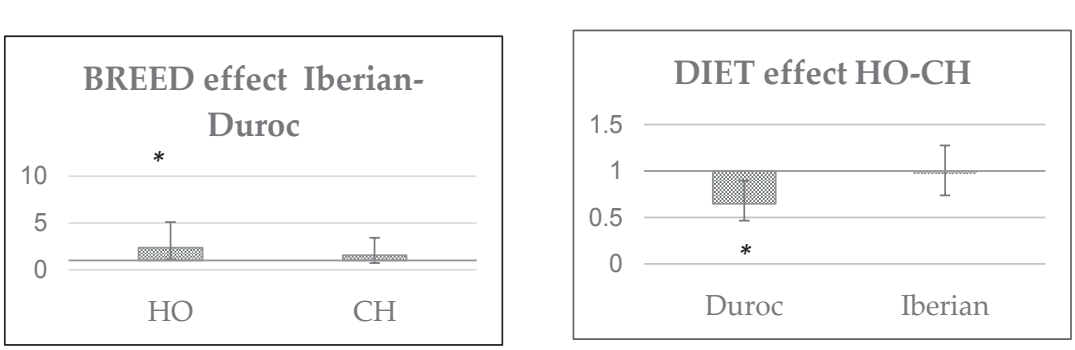
Breed*diet interaction *P*<0.0006

HSL



Breed*diet interaction *P*<0.07

PLIN1



Breed*diet interaction *P*<0.08

Figure S2. Diet*status interactions

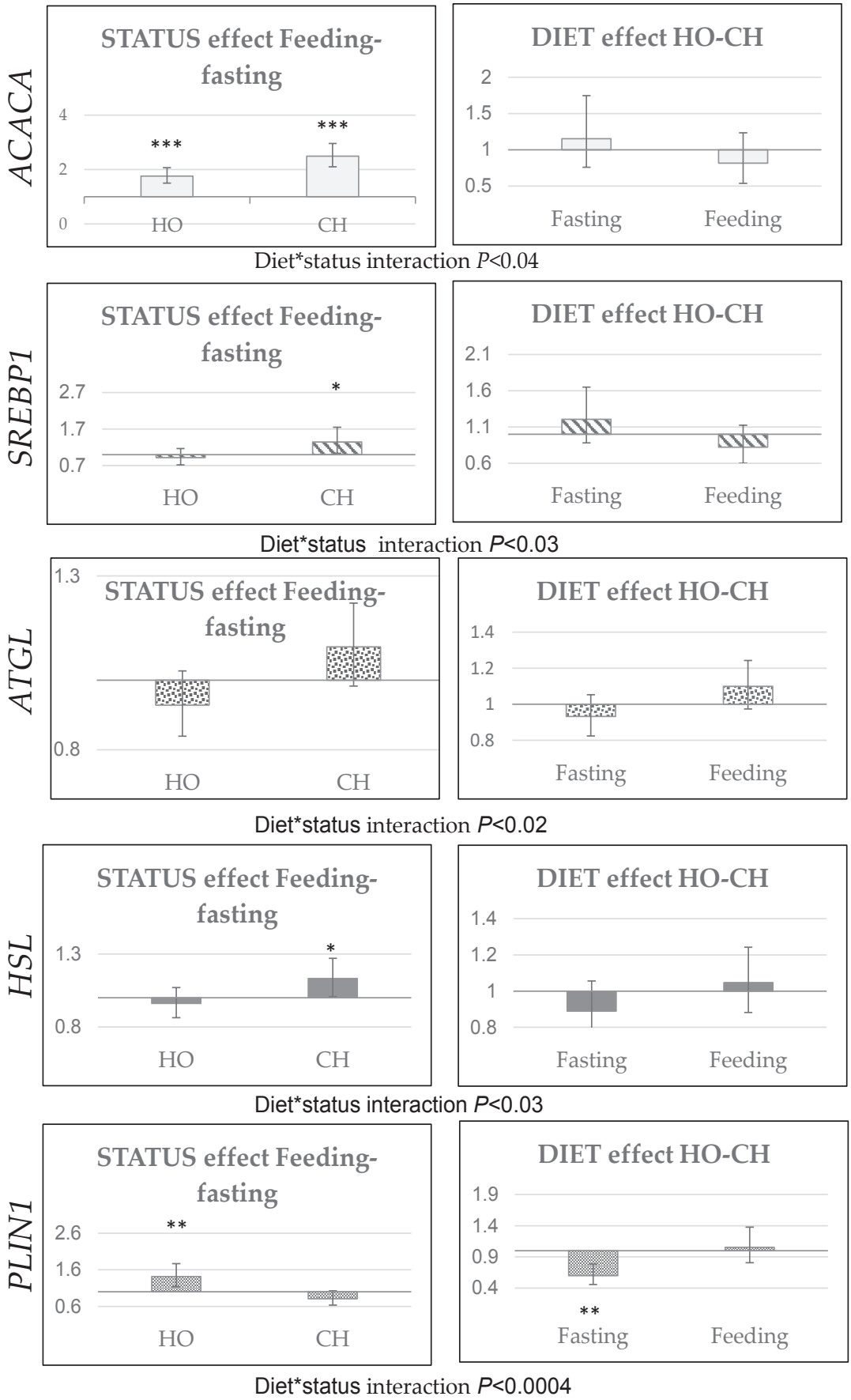


Table 4. Breed*status, breed*diet and diet*status interaction effects on candidate gene expression.

Gene	Breed*status	Breed*diet	Diet*status
	Nominal *P*-value	Nominal *P*-value	Nominal *P*-value
RXRG	ns	ns	ns
PPARG	ns	ns	ns
SREBP1	ns	ns	0.03
LEP	ns	ns	ns
ME1	0.07	0.07	ns
SCD	0.03	ns	ns
ACACA	0.0001*	ns	0.004*
FASN	ns	ns	ns
ELOVL6	ns	ns	ns
ATGL	ns	0.006	0.02
HSL	ns	0.07	0.03
PLIN1	ns	0.07	0.0004*
G0S2	ns	ns	ns

* significant adjusted P-values.

palmitic and stearic acids, indicating de novo synthesis of FA in this group from the available carbohydrates. PUFA content was also slightly affected by diet, and was higher in the HO group because of an increase in linoleic acid, in agreement with the diets' composition. All these results are coincident with previous works (Ventanas *et al*., 2008; Óvilo *et al*., 2014a) and are consistent with the deposition of dietary FA and the induction of lipogenesis by dietary CH. Different FA values and ratios are usually employed as measures of tissue metabolism and also of the tissue nutritional value in relation to human health. Particularly, fat saturation and high n-6 PUFA or n-6/n-3 ratio, are related to human diseases, such as atherosclerosis, cardiovascular disease, metabolic syndrome, cancer and autoimmune and inflammatory diseases (Simopoulos, 2002; Webb and O'Neil, 2008). The supplementation with high oleic sunflower oil reduced the saturation of pig fats and the n6/n3 ratio in both breeds, which positively affects the meat nutritional quality.

Most previous works of oleic acid supplementation were performed with Iberian type pigs and analyses were performed in backfat samples. The present results extend the findings to another breed not examined before, the cosmopolite Duroc breed, in which, according to our results, the employment of an oleic acid

supplemented diet may be a useful tool to improve the sensorial, organoleptic and nutritional quality of meat products. This finding is especially interesting in a breed which, in contrast to Iberian, has moderate meat quality attributes. Moreover, results have been validated in subcutaneous ham fat, which composition is essential for the production of the cured ham, the main pig cured product.

To the best of our knowledge, this is the first work in which purebred Iberian and purebred Duroc animals have been compared in identical environmental conditions. Results are relevant from a productive and scientific perspective. Main phenotypic traits related to fatness and premium cut yield (backfat thickness, ham weights and feed intake) strongly differed between breeds, as expected. Iberian pigs showed higher fatness and appetite and Duroc showed higher weight of the hams. The Iberian pig breed is a local fat breed characterized by an extreme adipogenic potential, rusticity and low productive efficiency, with bad conversion rates, low meat yield and low reproductive efficiency (López-Bote, 1998). Also, Iberian pigs are characterized by high levels of voluntary feed intake (Morales *et al*., 2002) in agreement with our findings. This high appetite is an adaptative mechanism of animals subjected to oscillations in food availability (thrifty genotype) (Ayuso *et al*., 2016; Óvilo *et* al., 2014a) and is concomitant with higher plasma levels of leptin hormone in comparison to other breeds (Fernández-Figares *et al*., 2007). Thus, in the Iberian breed high levels of leptin fail to reduce appetite, fitting with a pattern of obesity by leptin resistance.

Our Iberian and Duroc pigs clearly differed in fattening and appetite, however, it is interesting to note that according to our results, growing Iberian and Duroc pigs differed in ham weight, whereas no difference was found in body weight. Differences in ham measures between Iberian pig genotypes have been previously observed in juvenile and adult pigs in agreement with our work (Robina *et al*., 2023; Serrano *et al*., 2008). Although many studies have reported differences in weight between Iberian and Duroc genotypes at birth and at the final slaughter age, the differences in body weight and size between genotypes are not evident at weaning (Óvilo *et al*., 2014a) nor at the growing (Ayuso *et al*., 2016) periods, in concordance with the present findings. This fact has been associated with the thrifty genotype of Iberian pigs, which might led to high levels of voluntary feed intake and/or low energy expenditure during suckling and early growth periods,

without being reflected on adult pigs because of the lower growth potential of mature pure Iberian animals (Ayuso *et al*., 2016).

In the comparison between breeds, significant differences were also observed for FA composition. Jointly, higher SFA and lower PUFA is observed in Iberian compared to Duroc pigs; the changes are mainly due to a major increase of palmitic and stearic acids in Iberian pigs and an increase of linoleic acid in Duroc pigs. These results are in agreement with the greater de novo fat synthesis in the Iberian pig tissues, leading to a higher SFA content and a dilution effect of other FA. On the other hand, the level of PUFA in porcine tissues is only dependent on diet. Hence, the significantly higher levels of PUFA found in subcutaneous backfat and ham subcutaneous fat in Duroc pigs could be also explained by a higher ability of Duroc genotype to store dietary unsaturated lipids in their tissues (Ventanas *et al*., 2006).

3.3.4.2. Breed, feeding status and diet effects on candidate gene expression

The expression of a panel of adipogenic, lipogenic and lipolytic candidate genes was explored, allowing the identification of relevant breed and fasting effects on transcription, which gives insights into the differential regulation of lipid metabolism between a local obese and a cosmopolite breed. This work was only approached at the transcriptional level and thus the lack of activity information may be considered a limitation. However, gene expression measurements are considered reasonable measures of activity as a close relationship has been observed between both types of measurements (Ishii *et al*., 2004; Gosmain *et al*., 2005).

Breed had a considerable effect on gene expression, with genes involved in energy balance and lipogenesis being upregulated in Iberian. Leptin gene showed the highest upregulation in Iberian adipose tissue (2.85x), in agreement with the high leptin protein levels observed in Iberian pigs (Fernández-Figares *et al*., 2007), although this is the first report of the breed effect at the transcriptional level. The remaining genes upregulated in Iberian are directly involved in de novo lipogenesis in agreement with its phenotype. The *SCD* gene also showed a strong upregulation in Iberian samples (2.21x), matching with the findings of a previous muscle transcriptome comparison between Iberian and Duroc crossbred pigs (Óvilo

et al., 2014b). This *SCD* gene differential expression is also in agreement with the segrega*tion in our animals of a known polymorphism, AY487830:g.2228T>C in the* SCD gene promoter (Barea *et al*., 2013; Estany *et al*., 2014). This SNP has an allele (g.2228T) associated with a higher expression of the gene, which is fixed in our Iberian pigs but segregating in the Duroc ones. The *SCD* gene upregulation in Iberian may be associated with the higher desaturation potential of this breed (Fernández *et al*., 2017), although in our particular work this difference in gene expression is not translated in a higher MUFA content in Iberian pig tissues. One plausible explanation for this fact is the predominance of de novo lipogenesis over FA desaturation in the growing period analysed here, which may lead to intense increases in SFA and a dilution of the other FA. We did not detect any breed effect on transcriptional regulators involved in adipocyte differentiation (*RXRG*, *PPARG, SREBP1*), in contrast to previous findings in younger animals (García-Contreras *et al*., 2017), probably in agreement with the late moment of sampling regarding cellular differentiation processes. Jointly, the breed effects on lipogenic genes support the predisposition for obesity of Iberian breeds, provide a molecular basis for the development of leptin resistance and metabolic syndrome in Iberian pigs fed high fat diets (Torres-Rovira *et al*, 2012), and reinforces the usefulness of this pig as biomedical model for obesity and metabolic disorders.

Nine out of thirteen analyzed genes were significantly affected by feeding status, with all differentially expressed genes being upregulated in fed status. Specifically, differential expression was found for all the lipogenic genes (*SCD, ME1, ACACA, FASN, ELOVL6* and *LEP*), two regulators (*RXRG* and *PPARG*) and one lipolysis regulator gene (*GOS2*). Fasting, including short-term food deprivation, is known to reduce lipogenesis and induce lipolysis (Kersten, 2001; Palou *et al*., 2010; Duncan *et al*., 2010). Our results agree with fasting inhibiting lipogenesis and adipocyte differentiation. Nevertheless, regarding lipolysis, no response is observed after 24 h fasting on the main lipolytic genes *ATGL, HSL* and *PLIN1* and *GOS2* is activated after refeeding. Although this last gene is functionally involved in lipolysis, it is a negative regulator of lipolysis and previous studies have reported its downregulation after 24 h fasting, in concordance with our results (Zhang *et al*., 2014)

In our previous work Benítez *et al*., 2016, an 18 h fasting period affected the expression of the PPARG gene, which was activated after feeding, but had

no effect on downstream genes. The actual results confirm that this unexpected previous result was related, at least in part, to the duration of the fasting applied as a 24 h period has a much more intense effect on gene expression, in agreement with other works (Morgan *et al*., 2008; Houseknecht *et al*., 1998; O'Gorman *et al*., 2010). Thus, a medium-long food deprivation period (24 h of fasting) seems to be necessary to stop lipogenesis in these fatty animals. On the other hand, our previous results suggested that, in Iberian pigs, as previously proposed in obese mice, de novo lipogenesis may persist in adipose tissue during fasting (Morgan *et al*., 2008). In order to test the persistence of lipogenesis in Iberian breed during fasting, the breed and the fasting effects were considered jointly. Significant breed * feeding status interaction effects were observed for *SCD* and *ACACA* genes and a similar trend was observed for *ME1*. In the three cases, the interactions are quantitative and mean that the inhibition of these genes after 24 h fasting is more intense in Duroc pigs, in other words, its expression is more stable in Iberian pigs **(Figure 2)**. Moreover, the expression patterns of several other lipogenic genes (*SREBP1, LEP, FASN*) were similar, with a more pronounced effect of fasting in Duroc. Although the interaction effects for some genes did not reach statistical significance, jointly the results support a more persistent lipogenesis in Iberian than in Duroc breeds. This finding provides novel information on the regulation of fat deposition in genetically determined obesity versus previous scarce results only available for mice with diet induced obesity (Morgan *et al*., 2008). Moreover our results extend the findings to a large fasting (24 h) vs overnight fast and to new molecules not studied in the previous work as *SCD, ME1* and *LEP.*

Surprisingly, the diet did not affect the expression of any analysed gene but *PLIN1*, upregulated in the CH group. The scarce diet effects were unexpected as effects of similar diets were found in Iberian pigs in early and final growth stages (Óvilo *et al*., 2014a; Benítez *et al*., 2016). Nevertheless, the effects of FA dietary composition on gene expression are known to be scarce, small and difficult to measure and moreover these effects are deeply modulated by factors such as genotype or timing (Óvilo et al., 2014a). Thus, interaction effects may be conditioning our ability to detect and understand the diet effect on metabolism. In our previous work Benítez et al., 2016, we found evidence of a significant interaction diet * status on the *ME1* gene. In our actual results qualitative

interactions diet * status are observed for *ACACA, SREBP1, ATGL, HSL* and *PLIN1*. Regarding lipogenic genes, *ACACA* and *SREBP1*, the diet effects on gene expression seem to be opposite in fasting and feeding states, **(Figure S2).** For the lipolytic genes *ATGL, HSL* and *PLIN1* a similar qualitative interaction is found, with the same pattern for the three genes, but with an opposite trend to that of lipogenic ones. These interactions may be related to differences in the timing and availability of nutrients in blood after refeeding between both diets, as the CH diet is expected to increase glucose levels faster than the HO diet. Thus, for instance, the upregulation of lipogenic genes in CH group after refeeding is consistent with the expected major postprandial increase in serum glucose in animals fed a diet rich in carbohydrates.

In addition, a significant interaction breed * diet was found for the *ATGL* gene ($P<0.006$) and similar trends were found for *ME1, HSL* and *PLIN1* genes ($P<0.07$ for all) **(Figure S1)**. Although these results should be considered with caution due to the lack of statistical significance for some genes, the coincidence in the observed patterns for all the genes with a functional relationship allows suggesting an underlying biological basis, which should be explored in future works. Interestingly, the three main lipolytic genes, *ATGL, HSL* and *PLIN1*, present exactly the same pattern of diet * breed and diet * status interactions, in agreement with their common biological role. According to findings in mice and humans, the main lipases *ATGL* and *HSL* are downregulated in obese individuals (Duncan *et al*., 2010; Villena *et al*., 2004; Langin *et al*., 2005) and lipolysis is assumed to be impaired in obesity (Arner and Hoffstedt, 1999; Schiffelers *et al*., 2001; Luglio *et al*., 2015). In this study we would have expected to find lower lipolytic gene expression in Iberian breed and higher in fasted animals. The lack of significant main effects is related to the interactions breed * diet and diet * status. For example, for the ATGL gene downregulation in Iberian is only observed under the CH diet, and downregulation by fasting is only observed under the HO diet. Undoubtedly, all these complex interactions are hindering our ability to detect and interpret the influence of the different factors on gene expression.

Finally, it is known that a high level of leptin induces lipolysis at the adipose tissue level (Duncan *et al*., 2010); in our case leptin gene showed the highest upregulation in Iberian adipose tissue (2.85x) but we did not find a greater lipolysis in Iberian pigs. Leptin effects are dependent on the leptin receptor and

it is known that Iberian pigs show leptin resistance, related to the presence of polymorphisms in the LEPR gene that either reduce its expression or reduce its signaling ability (Óvilo *et al*., 2010; Pérez-Montarelo *et al*., 2013). Thus, the Iberian pigs would be resistant to leptin-induced lipolysis.

3.3.5. MATERIALS AND METHODS

3.3.5.1. Animals

The current study was carried out at the facilities of Pig Test Center ITACYL (Hontalbilla, Segovia, Spain). Animal manipulations were performed in compliance with the regulations of the Spanish Policy for Protection of Animals employed in Research and other scientific purposes RD53/2013, which meets the European Union Directive 2010/63/EU about the protection of animals used in experimentation. The study comprised a total of thirty Iberian and nineteen Duroc males born in nineteen contemporary litters. These animals were kept under identical management conditions. At ten weeks of age (SD=1.6 days), animals were distributed in two experimental groups and fed two different isocaloric and isoproteic diets (3.3 Kcal of digestible energy and 15.6% of crude protein) provided ad libitum and differing in the energy source: HO diet enriched with 6% high-oleic sunflower oil (17 Iberian and 10 Duroc pigs) and CH standard diet with carbohydrates as energy source (13 Iberian and 9 Duroc pigs). Fresh water was provided ad libitum. The animals started the experimental period at 19.9 kg (SD=3.8 kg) of average live weight (LW) and were slaughtered after 47 days of treatment, with 51.2 kg (SD=5.5 kg) of average LW. Batch feed intake was recorded daily. Feeds composition is shown in **Table 5.**

Four days before slaughter, ham subcutaneous fat samples were obtained in vivo by short-biopsies after a 24h-fasting period and 3h after refeeding (postprandial sampling). Tranquilization was performed by by intramuscular injection of 20 mg of azaperon per 10 kg live weight (Stresnil; Esteve, Barcelona, Spain) 1 h before micro-biopsies were taken. A cylindrical biopsy device with a diameter of 5 mm was employed for biopsies, which were taken under local anesthesia with 2 % lidocaine-HCL (Alphacaine; Fendigo, Brussels, Belgium). After procedure, the zone was treated with oxytretracycline and lidocaine (Veterin Tenicol; Lab. Intervet S.A., Salamanca, Spain). Pigs did not suffer any pain because of the

Table 5. Calculated analysis1 and fatty acid composition of experimental diets (g/kg, as-fed basis)

Diet	Carbohydrate (CH)[2]	High oleic (HO)[3]
Chemical composition, g/kg of feed		
Moisture	87.4	88.81
Lipids	24.53	77.65
Crude protein	156	156
Crude fiber	29.71	45.27
Nitrogen-free Extractives	515.75	404.39
Ash	44.34	67.91
Main Fatty acids, g/kg of feed		
C14:0	0.14	0.13
C16:0	4.83	7.19
C18:0	0.84	1.83
C18:1 n-9	9.47	36.82
C18:2 n-6	14.24	16.68
C18:3 n-3	0.99	1.21

[1] According to Fundación Española Desarrollo Nutrición Animal (2010); [2] CH = Carbohydrate diet without added fat; [3] HO = High oleic diet with high oleic sunflower oil

analgesia employed. Similar procedures have been used before (Raes *et al*., 2004; Serrano *et al*., 2008).

Biopsy samples were placed in cryotubes, snap frozen in liquid nitrogen and stored at −80°C until gene expression analyses .

Slaughter was performed at an experimental slaughterhouse (Hontalbilla, Segovia, Spain) after an overnight fasting period. Subcutaneous fat samples were collected from the carcasses at two levels: backfat at the last rib and ham fat, then fat was separated into outer and inner layers, which were separately analyzed for fatty acid composition.

Body weight, backfat thickness and hams weight were recorded at slaughter. Backfat thickness was measured in two locations, coinciding with the sampling points (loin and ham) with a ruler.

3.3.5.2. Tissue Composition Analyses

The samples of subcutaneous adipose tissue were kept frozen (-80ºC), until their analysis. Tissue lipid extracts were obtained by Bligh and Dyer method (Bligh

and Dyer, 1959). Samples were made in duplicate. All the samples were processed in the same day and their injection was done in less than a week, keeping the extracts at a temperature of -80ºC until their chromatographic injection. The precision of the fatty acid analyses measured as intra-day coefficient of variability (CV) was less than 5%. Fat extracts were methylated in the presence of sulfuric acid and analyzed by gas chromatography. Previously fatty acid methyl ester (FAME) samples were identified by gas chromatography as described elsewhere (López-Bote et al., 1997), using an HP-6890 (Hewlett Packard, Avondale, PA, USA) gas chromatograph equipped with a flame ionization detector and capillary column (HP-Innowax, 30 m by 0.32 mm i.d. and 0.25 µm polyethylene glycol-film thickness). A temperature program of 170 to 245°C was used. The injector and detector were maintained at 250°C. The carrier gas (helium) flow rate was 2 mL/min. For the identification of each fatty acid standard patterns were used (Sigma, Alcobendas, Madrid, Spain). Concentration of individual fatty acids was calculated as % of total fatty acids, results were expressed as grams per 100g of detected FAMEs. Response and corrections factors of each fatty acid are shown in **Table S1**.

Dietary FAs were extracted and quantified by the one-step procedure as described by Sukhija and Palmquist (Sukhija and Palmquist, 1988) in lyophilized samples. Pentadecanoic acid (C15:0) (Sigma, Alcobendas, Madrid, Spain) was used as internal standard. Previously methylated FA samples were identified by gas chromatography as described above. Results were expressed as grams per kg of feed.

3.3.5.3. Candidate gene expression analyses by quantitative PCR

The entire available adipose tissue sample (~50 to 100 mg) from each biopsy was used for total RNA extraction using RiboPureTM RNA isolation kit (Ambion, Austin, TX, USA) following the manufacturer's recommendations. RNA obtained was quantified using a NanoDrop equipment (NanoDrop Technologies, Wilmington, DE, USA) and RNA quality was assessed with an Agilent bioanalyzer device (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number values obtained for all the samples were in the range 7.5 to 8.5. First-strand cDNA synthesis was carried out with Superscript II (Invitrogen Life Technologies, Paisley, UK) and random hexamers in a total volume of 20 µl containing 1 µg of total RNA and following the supplier's instructions.

Selection of candidate genes.

We selected thirteen candidate genes. Sterol regulatory element binding transcription factor 1 (*SREBP1*) is recognized as the key transcriptional factor regulating lipogenic genes, Peroxisome proliferator activated receptor γ (*PPARG*), and Retinoic X receptor γ (*RXRG*) are involved in gene expression regulation of adipogenesis and lipogenesis processes (Kersten, 2001; Lefevbre *et al*., 2010). Leptin (*LEP*) encodes a hormone produced by adipose tissue, which controls energy balance and has local effects inhibiting lipogenesis in adipose tissue and promoting FA catabolism (Wang *et al*., 1999). In addition, five candidate genes with direct functional involvement in lipogenesis were selected: *ME1, SCD, FASN, ACACA* and *ELOVL6.* Malic enzyme 1 (*ME1*) encodes a cytosolic, NADP-dependent enzyme that generates NADPH for fatty acid biosynthesis (Stelmanska, 2007). Stearoyl-CoA desaturase (*SCD*) encodes an endoplasmic reticulum enzyme that catalyzes the biosynthesis of monounsaturated fatty acids from saturated fatty acids (Bernert and Sprecher, 1977). Fatty acid synthase (*FASN*) encodes an important enzyme that catalyses the biosynthesis of saturated fatty acids (SFA), mainly palmitic acid (Grzes *et al*., 2016) Acetyl-CoA carboxylase alpha (*ACACA*) encodes an enzyme that catalyzes the carboxylation of acetyl-CoA to malonyl-CoA a regulator of mitochondrial fatty-acid β-oxidation (Stachowiak *et al*., 2013). Fatty acid elongase 6 (*ELOVL6*) encodes an enzyme that catalyzes the rate-limiting step in the elongation cycle by controlling the fatty acid balance in mammals (Corominas *et al*., 2015) Moreover, four genes with functional involvement in lipolysis *ATGL, HSL, G0S2* and *PLIN1* were selected. Adipose triglyceride lipase (*ATGL*) and Hormone sensitive lipase (*HSL*) genes encode for two enzymes implicated in the complete hydrolysis of triacylglycerols (TAG) molecules in cellular lipid stores (Zechner *et al*., 2012; Lass *et al*., 2011). Under basal conditions, Perilipin 1 (*PLIN1*) restricts the access of cytosolic lipases (*ATGL* and *HSL*) to lipid droplets and thus promotes triacylglycerol storage. In times of energy deficit as fasting, *PLIN1* is phosphorylated by Protein kinase cAMP-dependent (PKA) and facilitates maximal lipolysis by *ATGL* and *HSL* (Brasaemle, 2007). The G0/G1 switch 2 (*G0S2*) gene is a negative regulator of lipolysis, which activation is known to downregulate *ATGL* expression. Further studies showed that 24 h fast down-regulated expression of *G0S2* and increased *ATGL* expression in adipose tissue (Ahn et al., 2013).

The candidate gene expression was quantified by qPCR. Primer pairs were designed using Primer Select software (DNASTAR, Wisconsin, USA) from the available GENBANK and/or ENSEMBL sequences. Primer pairs covered different exons for assuring the amplification of the cDNA. Information on primer sequences, efficiency and amplicon lengths are indicated in **Table 6.**

Standard PCRs on cDNA were carried out to verify amplicon sizes. Transcript quantification was performed using SYBR Green mix (Roche, Basel, Switzerland) in a LightCycler480 (Roche). The qPCR reactions were prepared in a total volume of 20 µl containing 2.5µl of cDNA (1/20 dilution), 10 µl of SYBR Green mix and 0.15µM of both forward and reverse primers. As negative controls, mixes without cDNA were used. Cycling conditions were 95°C for 10 min, followed by 45 cycles of 95°C (15 s) and 60°C (1 min) where the fluorescence was acquired. Finally, a dissociation curve to test PCR specificity was generated by one cycle at 95°C (15 s) followed by 60°C (20 s) and ramp up to 95°C with acquired fluorescence during the ramp to 0.01°C/s. Data were analyzed with LyghtCycler480 SW1.5 software (Roche). All points and samples were run in triplets as technical replicates and dissociation curves were carried out for each individual replicate. Single peaks in the dissociation curves confirmed the specific amplification of the genes. For each gene, PCR efficiency was estimated by standard curve calculation using four points of cDNA serial dilutions. Cycles to threshold (Cp) values were employed for the statistical analyses of differential expression. Data normalization was carried out by selecting the most stable endogenous genes out of GAPDH, ACTB, TBP, 18S, PPIA and B2M. Stability of endogenous genes was tested with Genorm software (Vandesompele *et al*., 2002) and Normfinder software (Andersen *et al*., 2004). ACTB and PPIA genes were employed for normalization.

3.3.5.4. Statistical analyses of tissue composition and candidate gene expression

The influence of breed and diet on fatty acid composition of carcass adipose tissue was analyzed with a linear model fitting breed, diet and the diet * breed interaction as fixed effects and litter and box as random effects. The influence of breed and diet on FA composition was separately analyzed for each FA or FA index in each tissue and layer. All the analyses were performed using MIXED procedure of SAS 9.1 (SAS Inst. Inc., Cary, NC, USA). Preliminary analyses were performed

Table 6: Primer design for qPCR and PCR efficiencies

Gene symbol	Gene name	GenBank ID	Forward primer sequence	Reverse primer sequence	Efficency (%)
RXRG	Retinoic X receptor gamma	DQ866834.1	GGGGTTGGCTCCATCTTTGA	ACCTGCCCGGCTGTTCTG	84.8
PPARG	Peroxisome proliferator activated gamma	NM_214379.1	GGCGAGGGCGATCTTGACAG	GATGCGAATGGCCACCTCTTT	93.5
SREBP1	Sterol regulatory element binding transcription factor 1	NM_214157.1	AGTTGAGCCCTGCCCCCGTGTTG	CTGCTGGATCTGCGAGGTCA	91.5
LEP	Leptin	NM_213840.1	GGCCCTATCTGTCCTACGTTGAAG	TGGAAGGCAGACTGGTGAGGAT	92.8
ME1	Malic enzyme	XM_001924333.4	GCCGGCTTTATCCTCCTCT	TCAAGTTTGGTCTGTATTTTCTGG	86.5
SCD	Stearoyl- CoA desaturase	NM_213781.1	TCCCGACGTGGCTTTTTCTTCTC	CTTCACCCCAGCAATACCAG	89.6
ACACA	Acetyl-CoA carboxylase alpha	NM_001114269.1	CTGAGAGCTCGTTTTGAAGGAATA	TTTACTAGGTGCAAGCCAGACAT	85.2
FASN	Fatty acid synthase	NM_001099930.1	GCAGGCGCGTGATGGGAATGGTG	GCCCGAGCCCGAGTGGATGAGCA	85.1
ELOVL6	Fatty acid elongase 6	XM_013978957.1	AGAACACGTAGCGACTCCGAAGAT	GACATGCCGACCGCCAAAGATAA	89.5
ATGL	Adipose triglyceride lipase	NM_001098605.1	GCACCTTCATTCCCGTGTAC	TTGTCTGAGATGCCACCGTC	87.2
HSL	Hormone sensitive lipase	NM_214315.1	CCCCCGTGCGCTGGAGGAGT	GGGAGGGGGAGGCGGCAGAC	82.8
PLIN1	Perilipin 1	NM_001038638.1	CCCCCTGGTGGCGTCTGTAT	ACTGGAGGGCCGGTATCTTTTCT	87.9
G0S2	G0/G1 switch 2	NM_001286804	GAGAGCCCGGAGCCGAGATGGA	CCGAGCACCGCGCCGAGAAA	83.7
ACTB	Beta-actin	XM_003124280.4	TCTGGCACCACACCTTCT	TGATCTGGGTCATCTTCTCAC	90.7
PPIA	Peptidylprolyl isomerase A	NM_214353	GGGAGAAAGGATTTGGTTAT	ATGGACAAGATGCCAGGAC	96.9

fitting the fat layer (outer and inner) and the interaction fat layer * diet in each breed.

The method proposed by Steibel *et al*. (Steibel *et al*., 2009) was employed for the statistical analysis of gene expression data. This procedure simultaneously analyses theCp values for the target and endogenous genes using a linear mixed model. The following model was used in a joint analysis of all the gene expression measures:

$$y_{gijklr} = TG_{gi} + P_{gj} + B_{gk} + A_{gl} + D_{ijkl} + e_{gijklr}$$

where $y_{gijklr} = -log_2\left(E_g^{-Cp_{gijklr}}\right)$ E_g brings the PCR efficiency for each gene, $C_{pgijklr}$ is the value obtained from the thermocycler software for the *g*th gene from the *r*th well in the *j*th qPCR plate and kth box corresponding to the lth animal subjected to the ith treatment, TG_{gi} is the specific effect of the ith treatment on the expression of gene g, P_{gj}, B_{gk} and A_{gl} are specific random effects on the expression in the *j*th qPCR plate of gene g, the *k*th box and the *l*th pig, D_{ijkl} is a random sample-specific effect common to all genes, and e_{gijklr} is a residual effect.

Eight different groups were fitted to the model: dietary effects (two levels: HO and CH) two breeds: (Iberian and Duroc pigs) and the four combinations of the two diets and fasting/feeding status in Iberian and Duroc pigs. To test differences or interaction between classes in the expression rate of genes of interest ($diff_{TG}$), different contrasts were performed between the appropriate estimates of TG levels. Significance of $diff_{TG}$ estimates was determined with the t statistic. Adjusted *P*-values were calculated using the correction method of Benjamini and Hochberg (Benjamini and Hochberg, 1995) for accounting the multiplicity of comparisons, with the Cheverud-Nyholt formula for the number of effective tests of Moskina and Schmidt (Moskina and Schmidt, 2008). Adjusted *P*-values for all analyses are indicated in the tables of results. Nevertheless, in order to perform a wide interpretation of the results, taking into account not only the significance of each individual analysis but the profiles and concordance of gene expression results for the genes grouped by functional category, results with nominal *P*-values < 0.05 were considered within the discussion. For this purpose the multiple test correction may be considered too demanding.

To obtain fold change values (FC) from the estimated $diff_{TG}$ values, the following equation was applied: $\boldsymbol{FC} = 2^{-diff_{TG}}$ Standard errors of fold change values (*SE*

(*FC*)) were calculated from the standard error of the estimated differences (*SE*), using a similar transformation: *SE* (*FC*) = $2^{\text{-SE}}$ Asymmetric confidence intervals (95% CI) were calculated for each FC value by using the SE values: 95% CI from $2^{-(diff_{TG}+t(\gamma,0.975).SE)}$ to $2^{-(diff_{TG}-t(\gamma,0.975).SE)}$, where t (*γ,0.975*) is the 97.5 quantile of the Student-*t* distribution with *γ* degrees of freedom. In our analyses γ ranged from 6 to 133.

3.3.6. CONCLUSIONS

The results of the present work provide a relevant phenotypic and transcriptional characterization of lipid metabolism processes in adipose tissue of pure Iberian and Duroc growing pigs bred and managed in identical conditions. Findings are of high scientific value and agree with the high lipogenic and desaturation potential, thrifty genotype and leptin resistance of the Iberian pig breed and support persistence of *de novo* lipogenesis during fasting in the obese Iberian pigs. A medium/long period of fasting is needed in this breed to induce an adaptation in lipogenic genes function. Joint results support the usefulness of the Iberian pig as animal model for obesity and metabolic disorders

On the other hand, lipolytic genes regulation in adipose tissue by breed, status and diet factors, scarcely studied to date, is much more complex, subjected to intricate interactions. The effects of FA profile of the diet on lipid metabolism are small and conditional on other factors. The joint results deepen in the knowledge of the molecular basis and regulation processes of lipid metabolism in both pig breeds and highlight the complexity of this regulation especially in relation to lipolysis processes.

Phenotipic characterization of the experimental groups provides novel and relevant information regarding the usefulness of supplemented diets to improve the pork quality, in both Iberian and Duroc pig breeds, with practical implications.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/link.

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Conflicts of Interest: "The authors declare no conflict of interest."

Abbreviations

HO	High Oleic diet
CH	Carbohydrates diet
FA	Fatty acid
SFA	Saturated fatty acid
MUFA	Monounsaturated fatty acid
PUFA	Polyunsaturated fatty acid
FC	Fold-Change
TAG	Triacylglycerols
PKA	Protein kinase cAMP-dependent

3. CAPITULO 4

Efectos de la raza, la dieta y la interacción en el transcriptoma del tejido adiposo de cerdos Ibéricos y Duroc alimentados con diferentes fuentes de energía.

Breed, diet and interaction effects on adipose tissue transcriptome in Iberian and Duroc pigs fed different energy sources.

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Breed, Diet, and Interaction Effects on Adipose Tissue Transcriptome in Iberian and Duroc Pigs Fed Different Energy Sources

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Abstract: In this study, we analyzed the effects of breed, diet energy source, and their interaction on adipose tissue transcriptome in growing Iberian and Duroc pigs. The study comprised 29 Iberian and 19 Duroc males, which were kept under identical management conditions except the nutritional treatment. Two isoenergetic diets were used with 6% high oleic sunflower oil (HO) or carbohydrates (CH) as energy sources. All animals were slaughtered after 47 days of treatment at an average live weight of 51.2 kg. Twelve animals from each breed (six fed each diet) were employed for ham subcutaneous adipose tissue RNA-Seq analysis. The data analysis was performed using two different bioinformatic pipelines. We detected 837 and 1456 differentially expressed genes (DEGs) according to breed, depending on the pipeline. Due to the strong effect of breed on transcriptome, the effect of the diet was separately evaluated in the two breeds. We identified 207 and 57 DEGs depending on diet in Iberian and Duroc pigs, respectively. A joint analysis of both effects allowed the detection of some breed–diet interactions on transcriptome, which were inferred from RNA-Seq and quantitative PCR data. The functional analysis showed the enrichment of functions related to growth and tissue development, inflammatory response, immune cell trafficking, and carbohydrate and lipid metabolism, and allowed the identification of potential regulators. The results indicate different effects of diet on adipose tissue gene expression between breeds, affecting relevant biological pathways.

Keywords: nutrigenomics; diet; breed; interaction; adipose tissue; Iberian pig; transcriptome; inflammation

1. Introduction

Most traits of interest for meat producers have a multifactorial background, with meat attributes being shaped by several genetic and environmental factors and their interactions. Within the environmental factors, feeding is the most important one in animal production. Nutrition influences animal body and tissue composition, and may be employed to improve carcass leanness, nutritional value of meat, as well as overall production efficiency [1].

In general, nutritional and genetics approaches, both in applied and scientific fields, have followed separate paths, ignoring how genome–nutrition interactions affect physiological and metabolic processes with important phenotypic consequences. From a metabolic perspective, quantitative and

3.4.1. ABSTRACT

In this study, we analyzed the effects of breed, diet energy source, and their interaction on adipose tissue transcriptome in growing Iberian and Duroc pigs. The study comprised 29 Iberian and 19 Duroc males, which were kept under identical management conditions except the nutritional treatment. Two isoenergetic diets were used with 6% high oleic sunflower oil (HO) or carbohydrates (CH) as energy sources. All animals were slaughtered after 47 days of treatment at an average live weight of 51.2 kg. Twelve animals from each breed (six fed each diet) were employed for ham subcutaneous adipose tissue RNA-Seq analysis. The data analysis was performed using two different bioinformatic pipelines. We detected 837 and 1456 differentially expressed genes (DEGs) according to breed, depending on the pipeline. Due to the strong effect of breed on transcriptome, the effect of the diet was separately evaluated in the two breeds. We identified 207 and 57 DEGs depending on diet in Iberian and Duroc pigs, respectively. A joint analysis of both effects allowed the detection of some breed–diet interactions on transcriptome, which were inferred from RNA-Seq and quantitative PCR data. The functional analysis showed the enrichment of functions related to growth and tissue development, inflammatory response, immune cell trafficking, and carbohydrate and lipid metabolism, and allowed the identification of potential regulators. The results indicate different effects of diet on adipose tissue gene expression between breeds, affecting relevant biological pathways.

Keywords: nutrigenomics; diet; breed; interaction; adipose tissue; Iberian pig; transcriptome; inflammation

3.4.2. INTRODUCTION

Most traits of interest for meat producers have a multifactorial background, with meat attributes being shaped by several genetic and environmental factors and their interactions. Within the environmental factors, feeding is the most important one in animal production. Nutrition influences animal body and tissue composition, and may be employed to improve carcass leanness, nutritional value of meat, as well as overall production efficiency (Raes et al., 2004).

In general, nutritional and genetics approaches, both in applied and scientific fields, have followed separate paths, ignoring how genome–nutrition interactions

affect physiological and metabolic processes with important phenotypic consequences. From a metabolic perspective, quantitative and qualitative properties of the diet components have important regulatory effects on muscle and lipid metabolism and influence gene expression (Loor et al., 2015). In this context, nutrigenomic studies make up a research field within nutritional sciences that allow us to elucidate how dietary nutrients can interact with genes affecting transcription factors, RNA and protein expression, cellular homeostasis, and metabolite production (Muller&Kersten, 2003).

Pork, along with poultry, is one of the most consumed meat worldwide. Nowadays, modern pig breeding is essentially based on highly-selected genotypes from lean breeds, which are managed in the framework of intensive production systems focused on the production of fresh pork meat. However, in the Mediterranean area these selected breeds coexist with local breeds, with Iberian pig being the most representative one. The Iberian breed has important commercial value because of its use for the production of high quality dry-cured products (López-Bote, 1998) and is characterized by its great appetite, high lipogenic potential, high desaturation capacity, and a distinctive fatty acid profile. These characteristics are due to its genetic predisposition (thrifty genotype) (Ayuso et al., 2016) and the traditional feeding system (acorns and pasture), which is a reference model for sustainable production of many local Mediterranean breeds (Pugliese, 2012). From the biomedical perspective, the specific physiological and metabolic characteristics of Iberian pigs, fitting a leptin resistance pattern, make them an adequate animal model for human metabolic and obesity studies (Cabot et al., 2001; Myers et al., 2008 and Walters et al., 2012).

Iberian pig production is based on both purebred Iberian and crossbred Duroc × Iberian pigs. These two genotypes show important phenotypic differences in growth, fattening, tissue composition, muscle differentiation, and several metabolic processes (Perez-Enciso et al., 2009 and Ayuso et al., 2016). Specifically, purebred Iberian animals show a lower lean growth efficiency and a higher meat quality than the Duroc × Iberian crossbreds (Ventanas et al., 2006; Óvilo et al., 2014 and Benítez et al., 2018).

The knowledge of the genetic mechanisms that regulate biological processes, such as muscle growth or fat deposition, is of great interest given their impact on production aspects and meat quality, as well as the scientific and translational

implications. In this sense, the study of differentially expressed genes in animals from different breeds and phenotypes or subjected to different types of diet may allow an increase in the knowledge of the molecular basis of nutrient effects on tissues and the associated phenotypic differences, potentially leading to the identification of genes and metabolic pathways directly involved in the regulation of the composition of tissues, and therefore in their quality.

In a previous work (Benítez et al., 2018), in order to better understand the effects of breed, fasting, and diet on candidate gene expression and the potential differential response between breeds, we studied pure Iberian and Duroc growing pigs, bred and managed in identical conditions, with a functional candidate gene approach focused on lipid metabolism genes. The results provided a relevant phenotypic and transcriptional characterization of lipid metabolism processes in these breeds and agreed with the differentiated metabolism of the Iberian pig breed. A main finding was the detection of quantitative interactions between breed and feeding status effects, showing a different response to fasting of the two breeds, with the fat Iberian breed showing a more stable expression of lipogenic genes after fasting (Benítez et al., 2018). Complex interactions were also observed in the expression of lypolitic genes. These previous results support the hypothesis of a differential response of both breeds to nutritional factors.

The present work employed the same experimental material as the quoted previous study (Benítez et al., 2018), with the objective of evaluating the effects of breed, diet (supplemented with 6% oleic sunflower oil (HO) or carbohydrates (CH) as energy source), and their interaction on subcutaneous ham fat transcriptome in growing Iberian and Duroc pigs, with a RNA-Seq approach.

3.4.3. MATERIALS AND METHODS

Ethics Statement. All experiments were performed in accordance with the regulations of the Spanish Policy for Protection of Animals employed in Research and other scientific purposes RD53/2013, which meet the European Union Directive 2010/63/EU on the protection of animals used in experimentation. The project was approved on March 20, 2015, by the Comunidad de Madrid animal welfare and protection committee (reference number PROEX-007/15).

Animals and Sampling. The current study was carried out at the facilities of the Instituto Tecnológico Agrario de Castilla y Leon (ITACYL) Pig Test Center

(Hontalbilla, Segovia, Spain). The study comprised a total of 29 Iberian Torbiscal and 19 Duroc males born in 19 contemporary litters, which started the experiment at 19.9 kg (standard deviation (SD) = 3.8 kg) average live weight (LW). These animals were kept under identical management conditions, housed in batches of 4 pigs/pen (1 m^2 pig^{-1}), with a concrete floor and straw bedding. Temperature was controlled at a mean of 23.8 °C throughout the experiment. At 10 weeks of age (SD = 1.6 days), the animals were distributed in two experimental groups and fed two different isocaloric and isoproteic diets (3.3 kcal digestible energy and 15.6% crude protein) provided for ad libitum consumption and differing in the energy source: HO diet contained 6% high oleic sunflower oil (17 Iberian and 10 Duroc pigs), and CH diet was formulated to contain carbohydrates as energy source (13 Iberian and 9 Duroc pigs). Feed composition is shown in Table S1. Fresh water was provided ad libitum, with two drinking troughs available in each pen. The animals were slaughtered after 47 days of treatment, at an average LW of 51.2 kg (SD = 5.5 kg).

The animals were sampled immediately after euthanasia, which was performed by electrical stunning and exsanguination in compliance with RD53/2013 standard procedures. Subcutaneous ham fat samples were collected from the carcasses. The fat was separated into outer and inner layers and samples were preserved at −80 °C. The inner layer was used for RNA extraction and gene expression analyses.

RNA Isolation, Library Construction, and Sequencing. For the transcriptomic study, 24 animals were used (6 animals of each breed, corresponding to each diet group). Total RNA was isolated from 50–100-mg samples of subcutaneous ham fat using the RiboPureTM RNA isolation kit (Ambion, Austin, TX, USA), following the manufacturer's recommendations. The obtained RNA was quantified using NanoDrop equipment (NanoDrop Technologies, Wilmington, DE, USA), and the RNA quality was assessed with an Agilent 2100 bioanalyzer device (Agilent Technologies, Palo Alto, CA, USA) and submitted to the Centro Nacional de Análisis Genómico (CNAG-CRG; Barcelona, Spain). Libraries were prepared using the TruSeq mRNA-Seq sample preparation kit (Illumina Inc., Cat. # RS-100-0801, San Diego, CA, USA) according to the manufacturer's protocol. Each library was paired-end sequenced (2 × 75bp) by using TruSeq SBS Kit v3-HS in a HiSeq2000 platform (Illumina, Inc.).

Bioinformatic Analyses. FastQC [14] was used to assess the quality of raw sequencing data. TrimGalore [15] was used to qualitatively trim data with default settings and to remove the sequencing adaptors and poly A and T tails (stringency of 6 bp, -s 6), keeping only paired-end reads where both pairs were longer than 40 bp. Filtered reads were mapped against the pig reference genome (Sscrofa11.1) using TopHat v.2.1.0 (Kim et al., 2013) with Bowtie2 (v.2.2.7.0), applying default settings, except that the reads were first aligned to the ENSEMBL (11.1.90) (zerbino et al., 2018) transcriptome annotation (-G option), the distance between both pairs was set to 100 bp (inner-mean distance), and the standard deviation to 150 bp.

In order to confirm that the mapping had been carried out correctly, a quality control was performed in all samples using two tools, Samstats (Lasmann et al., 2010) and Qualimap (Okonechnikov et al., 2015). These programs provide information on the quality score of each mapped read, its length, and depth of mapping, composition, and quality of the bases.

For the first employed pipeline, transcripts were assembled using Cufflinks (v2.2.1.), following the protocol proposed by the developer and including the Cufflinks, Cuffmerge, and Cuffdiff steps (Tranpnell et al., 2012); transcript abundances were estimated as fragments per kilobase of transcript per million (FPKM) mapped reads. A filtering of the DEGs obtained was carried out following three criteria: an average expression greater than 0.5 FPKM in at least one of the groups, a fold change value (FC) ≥ 1.5 and a false discovery rate (FDR) < 0.05 for the breed effect, and FDR < 0.1 for the diet effect, the same as in previous studies (Ayuso et al., 2016).

Differential expression analyses were also carried out in parallel with an R package, after raw counts for the genes and transcripts were obtained with HTSeq-counts (Anders et al., 2015) (https://pypi.python.org/pypi/HTSeq). DESeq2 (Love et al., 2014) was used at an FDR adjusted q-value ≤ 0.05 for the breed effect and q-value ≤ 0.1 for the diet effect and FC ≥ 1.5. This software supports more complex experimental designs in addition to simple two-group setups. With DESeq2 software, RNA-Seq read counts were modeled by generalized linear models, including the breed and diet effects, the diet effect within each breed, and with a full model including breed, diet, and the breed–diet interaction effects.

Results Validation by Quantitative PCR (qPCR). RNA obtained from the 24 animals used in the RNA-Seq assay was used to perform the technical validation

of the differential expression of 11 genes that were either affected by the breed or the diet within each breed. This technical validation was performed by studying the Pearson correlation between the expression values obtained from RNA-Seq data (FPKM) and the normalized gene expression data obtained by RT-qPCR. To validate the global RNA-Seq results, the concordance correlation coefficient (CCC) (Miron et al., 2006) was calculated between the FC values estimated from RNA-Seq and qPCR expression measures for the 11 genes analyzed by the two technologies. Moreover, RNA obtained from all 48 available animals (29 Iberian and 19 Duroc) was used to quantify expression differences by qPCR and provide biological validation. The method proposed in previous work (Steibel et al., 2009) was employed for the statistical analysis of qPCR gene expression data, following the procedure explained in another study (Benítez et al., 2018). The p-values < 0.05 were considered statistically significant.

The expression of the genes *PCK1, PLIN2, IGFBP3, JAZF1, PDLIM3, PYGM, RBP7, ASB2, EEF1A2, SERPINE1*, and *CYP1A1* was quantified employing the method previously described. Primer pairs were designed using Primer Select software (DNASTAR, Madison, WI, USA) from the available GENBANK or ENSEMBL sequences. Primer pairs covered different exons to assure the amplification of the cDNA. Information on primer sequences, efficiency, and amplicon lengths are indicated in Table S2. The most stable endogenous genes out of *GAPDH, ACTB, TBP, 18S, PPIA*, and *B2M* were selected for data normalization. The stability of the endogenous genes was tested with the Genorm and the Normfinder software (Vandesompele et al., 2002 and Andersen et al., 2004). Thus, ACTB and PPIA genes were selected as endogenous genes.

Functional Interpretation. Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) software was used to identify and characterize biological functions, gene ontologies, canonical pathways, and regulatory networks affected by the DE genes. The IPA Canonical Pathways Analysis identified in its library the pathways that were most significant in our dataset. The significance of the association between the dataset and the canonical pathway was measured with Fischer's exact test. IPA software transforms a set of genes into a number of relevant networks based on comprehensive records maintained in the Ingenuity Pathways Knowledge Base. Networks are presented as graphs depicting the biological relationships between genes. Complementarily,

IPA software has a tool used to identify and characterize potential regulators (upstream regulators and causal networks). This tool identifies known regulators, including genes and other molecules that may affect the expression of DE genes. The analysis of upstream regulators considers every possible transcription factor and upstream regulator contained in the Ingenuity Knowledge Base repository, as well as their predicted effects on gene expression. Then, this tool analyzes whether the patterns of expression observed in the DEGs can be explained by the activation or inhibition of any of these regulators through the calculation of a z-score, a statistical measure of the match between the expected relationship direction between the regulator and its targets, and the observed gene expression (Krämer et al., 2014).

3.4.4. RESULTS AND DISCUSSION

Iberian and Duroc pig genotypes differ in growth, fatness, and meat composition and properties (Ventanas et al., 2006, Olivares et al., 2010 and Fuentes et al., 2014), even from early developmental stages (Óvilo et al., 2014b and Ayuso et at., 2016). Specifically, a phenotypic characterization of the same Iberian and Duroc pure animals employed here has been previously reported (Benítez et al., 2018). Briefly, the Iberian growing pigs showed greater average feed intake, backfat thickness, and saturated fatty acid (SFA) content, whereas the Duroc pigs had greater ham weight and polyunsaturated fatty acid (PUFA) content. Regarding the diet composition (oleic acid versus carbohydrates), no effect was observed on growth or fattening traits, but HO group showed a higher MUFA content in adipose and muscular tissues, while the CH group showed a higher SFA content (Óvilo et al., 2014 and Benítez et al., 2018), with results indicating the direct deposition of MUFA in HO group and the activation of de novo lipid synthesis from carbohydrates in CH group.

In the present work the ham subcutaneous adipose tissue (AT) transcriptome of 24 animals was characterized with RNA-Seq in order to study the effects of breed, diet, and breed–diet interaction. An average of approximately 50 million sequence reads was obtained for each individual sample and was assembled and mapped to the annotated Sscrofa11.1 genome. All samples passed the quality control and 91–93% of the reads were mapped to the porcine reference sequence. An average of 19,181 genes out of 22,452 annotated genes were expressed in

the studied samples. Regarding mapping quality values (MAPQ), an average of 96% of the reads showed MAPQ ≥ 30 in the different samples (which correspond to a probability of a correct match equal to or higher than 0.999).

The differential expression analysis was performed using two pipelines, a standard protocol proposed in a previous study (Trapnell et al., 2012), involving Cufflinks, Cuffmerge, and Cuffdiff tools, another one employing HTSeq–counts to construct the read counts matrix (Anders et al., 2015), and DESeq2 for the differential expression analyses (Love et al., 2014). This second pipeline uses a design formula that includes additional variables and allows the employment of complex models with more than one factor influencing the read counts, thus allowing us to test the interaction effect.

In order to validate the results obtained from the RNA-Seq analysis, the relative expression of 11 genes was assessed by qPCR in all available samples (n = 48). Genes for validation were selected from the lists of DEGs affected by the breed, diet, and interaction effects obtained in the different pipelines. For the technical validation we calculated the Pearson correlation values between RNA-Seq and qPCR data, which showed significant results in all cases (Table 1, correlation values ranging from 0.63 to 0.96, p-values ranging from 0.004 to 2.42 × 10−6). Moreover, the CCC coefficient, used to assess technical validation in high throughput transcriptomic studies, was calculated and a value of 0.81 was obtained, denoting a substantial general concordance between RNA-Seq and qPCR expression values (Miron et al., 2006). Biological validation was performed by analyzing the different tested effects with the qPCR data obtained from all available animals (n = 48). Regarding the breed effects, six out of seven DEGs detected with RNA-Seq had confirmed significance in the qPCR analysis. For the diet effect, all four selected DEGs affected by diet in Iberian pigs according to RNA-Seq were also significant after qPCR analysis. Moreover, three additional genes were detected as significantly affected by diet in Iberian pigs by qPCR, which were not initially detected with RNA-Seq (*PCK1, JAZF1*, and *PDLIM3*). For Duroc pigs, five DEGs depending on diet were selected for validation, and in this case three of these DEGs were confirmed by qPCR, although one of them only showed suggestive significance value (*CYP1A1*, p-value = 0.09; Table 1). Again, we detected DEGs affected by diet in Duroc with the qPCR analysis, which were not detected previously with RNA-Seq (*IGFBP3, JAZF1,* and *RBP7*). The detection of additional DEGs in the qPCR step may be

Table 1. Technical and biological validation of RNA-Seq results by quantitative PCR (qPCR): genes, statistical significance, and fold change values (FC) obtained with both techniques for the breed, diet, and interaction effects, and Pearson correlations between expression values obtained from both techniques.

	BREED EFFECTS (Ib *vs* Du)				DIET EFFECTS (HO *vs* CH)								INTERACTION		CORRELATION	
					IBERIAN				DUROC							
GENES	RNA Seq (n=24)		qPCR (n=48)		RNA Seq (n=12)		qPCR (n=29)		RNA Seq (n=12)		qPCR (n=19)		DESEQ2	qPCR		
	q-value	FC	*p*-value	FC	*q*-value	FC	*p*-value	FC	*q*-value	FC	*p*-value	FC	*p*-value	*p*-value	Correlation (r)	*p*-value (H0: r=0)
PCK1	0.001	1.81	0.0005	2.76	0.89	1.11	0.003	1.97	0.99	1.13	0.31	1.38	0.99	0.02	0.81	3.47E-06
PLIN2	0.001	1.55	<.0001	5.02	0.99	0.92	0.63	1.08	0.49	0.91	0.51	0.86	0.99	0.42	0.64	0.001
IGFBP3	0.001	1.80	<.0001	5.51	0.007	2.57	0.02	1.61	0.90	1.34	0.001	1.31	0.25	0.1	0.68	4.00E-04
JAZF1	0.90	1.71	0.066	1.10	0.99	0.71	<.0001	0.01	0.90	1.02	0.01	1.03	3.51E-06*	<.0001	0.79	1.23E-05
PDLIM3	0.001	0.11	0.001	0.13	0.85	0.82	0.02	0.44	0.02	2.78	0.02	1.58	0.19	0.76	0.63	0.002
PYGM	0.001	0.75	0.5	0.67	0.91	0.89	0.28	0.48	0.08	3.32	0.07	1.31	0.20	0.86	0.65	0.001
RBP7	0.001	0.46	<.0001	0.58	0.87	0.84	0.21	0.61	0.99	1.15	0.05	1.23	0.15	0.05	070	0.0002
ASB2	0.35	0.07	0.18	0.1	0.90	0.94	0.31	0.59	0.02	3.23	0.8	1.15	0.41	0.81	0.92	5.46E-05
EEF1A2	0.02	0.01	0.05	0.10	0.02	4.49	0.001	6.66	0.02	3.86	0.11	2.14	0.80	0.04	0.96	2.42E-06
SERPINE1	0.19	1.27	0.11	1.51	0.02	1.61	0.01	1.36	0.02	0.56	0.004	0.89	0.001	0.003	0.77	0.004
CYP1A1	0.95	0.95	0.11	0.39	0.008	2.14	0.03	2.97	0.02	3.85	0.09	1.34	0.005	0.02	0.70	0.001

Ib: Iberian; Du: Duroc, HO: high oleic diet; CH: carbohydrate diet
*Statistically significant at *q*-value<0.05

indicative of a higher precision of qPCR with respect to RNA-Seq, but mainly reflects the results of a larger sampling (n = 48 for qPCR, instead of the 24 total animals employed for RNA-Seq, which are reduced to 12 when the diet effect is explored within breed). The interaction effects breed–diet were confirmed for six genes, twice more than those selected from RNA-Seq data. Out of these, four showed quantitative interactions (*EEF1A2, CYP1A1, RBP7*, and *PCK1*) and two genes (*JAZF1* and *SERPINE1*) showed qualitative interactions, with opposite diet effects in both breeds. For *JAZF1* gene we detected higher expression in CH group in Iberian pigs, but in HO group in Duroc pigs. For *SERPINE1*, in contrast, we observed higher gene expression in HO diet in Iberian pigs, but in CH diet in Duroc pigs.

3.4.4.1. Breed Effect on Transcriptome

Cufflinks pipeline identified 837 DEGs between breeds (FC ≥ 1.5 and FDR < 0.05). DESeq2 detected as many as 1435 DEGs after the application of the same significance and magnitude thresholds (Table S3). This software allows the analysis of complex models including several effects, and thus the breed effect was also tested including the diet effect in the model, yielding 1456 DEGs. Out of the DEGs affected by breed in the two methods, 602 were common (Table S3). Taking into account the high number of DEGs detected with the tested methods, which allowed us a subsequent functional analysis, we used the DEGs obtained with Cufflinks for functional interpretation, despite being a conservative option.

Cufflinks detected 337 genes upregulated in Iberian pigs, with FC ranging from 1.5 to 45, and 500 DEGs upregulated in Duroc with FC ranging from 1.5 to 271. The genes showing the largest expression differences between breeds were *CLCA1* (*chloride channel accessory* 1, FC = 45, upregulated in Iberian) and *PVALB* (*parvalbumin,* FC = 271, upregulated in Duroc). *CLCA1* gene may act as an innate immune signaling molecule that activates macrophages, and thereby enhances pro-inflammatory cytokine release (IL-8, IL-6, IL-1β, TNF-α) and has an impact on the early innate immune response in mice (Dietert et al., 2014). Parvalbumin (*PVALB*) is a member of the EF-hand superfamily (helix-loop-helix structural motif) expressed in vertebrates in a tissue- and cell-specific manner, serving as a magnesium/calcium buffer. EF-hand proteins are involved in a variety of physiological processes, including cell-cycle regulation, second

messenger production, muscle contraction, microtubule organization, and vision (Cates et al., 1999 and Permyakov et al., 2017).

As expected, genes upregulated in Iberian pigs included several known biological candidate genes involved in lipid metabolism and energy homeostasis, such as *LEP*, *PCK1*, *RXRG*, *IGBP3*, *RBP7*, *ME1*, *FADS2*, or *PLIN2*. Higher expression of *LEP* (FC = 2.13) in Iberian AT is in agreement w ith previous findings (Fernández-Figares et al., 2007 and Benítez et al., 2018). Leptin is a hormone mainly produced by adipocytes, whose role is the control of energy balance at the hypothalamic level and also has local effects in peripheral tissues where it inhibits lipogenesis and promotes FA catabolism (Ceddia et al., 2001). Obesity is associated with leptin production and high plasma leptin concentration (Ahima, 2006), thus, an increased expression of *LEP* gene is expected in animals with increased fat deposition, as has been observed in the fatty Iberian pig (Fernández-Figares et al., 2007 and Benítez et al., 2018). Cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) (FC = 1.81) is also an interesting candidate gene due to its involvement in lipid and carbohydrate metabolism. It is one of the main regulatory enzymes of gluconeogenesis and glyceroneogenesis (Hanson&Reshefd., 2003), providing glycerol-3-phosphate as a precursor for fatty acid esterification in triglyceride synthesis. In fact, this gene has been recently associated with fat deposition in different mammal species, and a missense SNP has been identified to underlie this association in pigs (Latorre et al, 2016). Allele *PCK1* c.2456A, associated with higher enzyme activity and better meat quality, is almost fixed in Iberian pigs (Muñoz et al., 2018) and is fixed in the Torbiscal strain employed in this work. Our results show that, besides higher *PCK1* activity due to the polymorphism, Iberians are characterized by higher *PCK1* expression, supporting further increases in fat deposition in this breed, in agreement with the phenotype. Malic enzyme (*ME1*) gene is an essential gene directly involved in de novo lipogenesis that encodes a cytosolic enzyme that generates NADPH (Dihydronicotinamide-adenine dinucleotide phosphate) for fatty acid biosynthesis (Stelmańska, 2007). Higher expression of *ME1* gene (FC = 1.5) in Iberian versus Duroc pigs is in agreement with our previous results obtained by qPCR in ham subcutaneous fat samples (Benítez et al., 2018) and with the known higher lipogenesis of our fat breed (Lopez-Bote., 1998 and Fernández-Figares et al., 2007).

Among the genes upregulated in Duroc we found the *IGF2* (FC = 2.67) gene, which encodes for a member of the insulin family of polypeptide growth

Table 2. Ingenuity Pathway Analysis (IPA) -based list of pathways in the set of differentially expressed genes (DEGs) according to breed (*p*-value < 0.01, z-score > 2 or <−2).

Canonical Pathways	*p*-value	Ratio [1]	z-score [2]	Molecules
Glutathione Redox Reactions I	0.008	4/24	-2	*GPX3,MGST2,GPX1,GSTP1*
LXR/RXR Activation	0.0002	13/121	-1.155	*C3,MSR1,RXRG,LYZ,TF,NGFR,S100A8,PTGS2,LBP,PON3,CLU,TNFRSF11B,RBP4*
Chemokine Signaling	0.005	8/80	1.134	*FOS,RASD2,CCL4,CAMK2A,MYL2,PIK3C2G,CCL11,CAMK2B*
Acute Phase Response Signaling	0.007	13/179	1.134	*RASD2,C3,RBP1,FOS,RBP7,AKT1,TF,NGFR,LBP,CRABP2,A2M,RBP4,TNFRSF11B*
Ceramide Signaling	0.008	9/104	1.134	*S1PR3,FOS,CTSD,RASD2,AKT1,CNKSR1,NGFR,PIK3C2G,TNFRSF11B*
Endocannabinoid Neuronal Synapse Pathway	0.01	10/128	1.265	*CACNG6,CACNB1,CACNA1S,CACNG1,ADCY2,GRIA2,PTGS,ADCY10,PLCH1,GRIN3A*
TGF-β Signaling	0.01	8/96	1.633	*FOS,RASD2,TGFB3,BMP7,HNF4A,INHBA,INHBB,PMEPA1*
GP6 Signaling Pathway	0.002	12/135	1.732	*COL8A2,AKT1,COL6A1,COL12A1,LAMB1,PIK3C2G,COL22A1,COL8A1,COL11A1,COL11A2,LAMC2,COL26A1*
Synaptogenesis Signaling Pathway	0.003	21/32	1.886	*THBS4,ADCY2,CACNB1,RASD2,CACNG1,CDH18,PIK3C2G,GRIA2,GRIN3A,EPHB6,CDH1,SYT13,AKT1,CAMK2A,THBS1,DLG4,NECTIN1,SYT2,ADCY10,SYT12,CAMK2B*
ILK Signaling	0.001	16/205	2	*MYH4,MYL2,ACTN2,DIRAS3,ACTN3,PIK3C2G,MYH7,ITGB8,MYL1,FOS,CDH1,RHOQ,AKT1,MYH2,PTGS2,ACTG2*
Actin Cytoskeleton Signaling	0.002	17/234	2.111	*MYH4,RASD2,MYL2,ACTN2,MYLPF,MYLK2,ACTN3,PIK3C2G,EGF,MYH7,MYL1,MYH2,FGF18,LBP,ACTG2,NCKAP1L,MATK*
Opioid Signaling Pathway	0.01	16/250	2.138	*CACNG6,ADCY2,CACNA1S,CACNG1,RASD2,CACNB1,RGS3,RGS7,PIK3C2G,GRIN3A,FOSB,FOS,CAMK2A,AKT1,ADCY10,CAMK2B*
Cardiac Hypertrophy Signaling (Enhanced)	0.0007	31/498	2.502	*ADRA2B,IL15RA,CACNA1S,RASD2,LEP,PLCH1,ATP2A1,CAMK2A,NFAT5,AKT1,FGF18,NGFR,WNT4,TNFSF15,CAMK2B,IL11RA,TNFRSF11B,SMPDL3A,HDAC9,ADCY2,IL15,PIK3C2G,IL20RB,ADRA2A,TGFB3,IL2RA,HSPB7,PTGS2,ADCY10,HSPB1,WNT5A*
Calcium Signaling	0.00001	24/206	2.887	*HDAC9,CACNG6,MYH4,TNNT1,CACNB1,CACNG1,CACNA1S,MYL2,TNNI2,TNNT3,TNNC2,GRIA2,MYH7,TPM1,TPM2,MYL1,ATP2A1,ATP2B2,GRIN3A,MYH2,CAMK2A,NFAT5,TNNI1,CAMK2B*

[1] Ratio= number of DEGs in a pathway divided by the number of genes comprised in the same pathway.
[2] Positive z-scores predict an overall increase in the activity of the pathway in Duroc pigs, while negative z-scores indicate a prediction of an overall increase in the pathway activity in Iberian.

factors that involved in development and growth. It is a paternally imprinted gene, associated with fat deposition, muscle growth, and heart size. A causal mutation in *IGF2* intron3 (g.3072G>A) has been detected in pigs (Van Laere et al., 2003; Burgos et al., 2012) which influences production and carcass traits, with *IGF2* g.3072G allele having strong adipogenic effects at the subcutaneous AT level. This mutation shows different alleles in the two analyzed breeds—a high frequency of the mutant *IGF2* g.3072A allele is observed in Duroc breed, while it is almost absent in Iberian pigs (Alves et al., 2012; Muñoz et al, 2018). The mutation is associated with increased postnatal *IGF2* expression (Van Laere et al., 2003) and thus upregulation of *IGF2* in Duroc genotypes is in agreement with previous evidence and with the presence of alternative alleles in both analyzed breeds.

Besides the individual interpretation of selected candidates, global gene expression differences were functionally interpreted. Forty-nine canonical pathways were significantly enriched (p-value < 0.01) in the dataset of 837 DEGs (Table S4). Moreover, 26 pathways were assigned a z-score value and 6 were predicted to be significantly activated or inhibited (z-score > 2 or <−2, Table 2). Regarding gene ontology (GO) enrichment, 249 biological functions were predicted to be affected by breed (p-value < 0.01) (Table S5). Out of them, 21 were significantly activated or inhibited (z-score < −2 or >2).

For instance, pathways enriched in Iberian pigs included *LXR/RXR* activation, which is involved in the regulation of lipid metabolism, inflammation, and cholesterol metabolism (Murthy et al., 2002), glutathione redox reactions, which are related to balance of reduction/oxidation (redox) state of the cell (Filomeni et al., 2002), or *p53* signaling, which plays an important role in the coordination of the cellular response to different types of stress, including oxidative stress (Liu and Xu, 2010). In agreement, the biological functions enriched in AT from Iberian pigs (Table S5) were mainly related to inflammatory response (i.e., inflammatory response, chronic inflammatory disorder, binding of leukocytes, cell movement of leukocytes, granulocytes, and myeloid cells and adhesion of immune cells), and lipid and carbohydrate metabolism (i.e., synthesis of lipid and eicosanoid, fatty acid metabolism, homeostasis of D-glucose, metabolism of carbohydrates, diabetes mellitus). Significant activation (z-score < −2) was only observed for functions related to inflammation (binding of leukocytes and adhesion of immune cells).

Table 3. IPA-based list of activated upstream regulators (sorted by z-score) in the set of DEGs according to breed (*p*-value < 0.01 and z-score > 2 or <−2).

Upstream Regulator	Expression Log Ratio(Du/Ib)	Molecule Type	Activation z-score [1]	*p*-value of overlap	Molecules in dataset	Related functions
ACTIVATED IN IBERIAN						
KDM5A		transcription regulator	-3.357	3.28E-05	15	Epigenetic regulation of inflammation
DNMT3A		enzyme	-3.051	2.74E-06	16	Inflammation and lipid metabolism
AHR		ligand-dependent nuclear receptor	-2.702	9.15E-10	34	Inflammatory and immune response
SMTNL1		other	-2.668	2.13E-08	10	
PTEN		phosphatase	-2.569	0.003	21	Cell migration, survival and proliferation
HNF4A	1.212	transcription regulator	-2.53	5.71E-05	33	Inflammation
NR1H3		ligand-dependent nuclear receptor	-2.294	0.01	13	Inflammation and fat cell metabolism
NOS2		enzyme	-2.145	8.99E-04	15	Inflammation and insulin resistance
MED1		transcription regulator	-2.138	0.001	12	Activation of gene transcription
RBPJ		transcription regulator	-2.101	0.0002	13	Polarization of macrophages
ACTIVATED IN DUROC						
Bvht		other	3.148	2.16E-04	10	
ZNF106		other	3	1.68E-05	9	
INSIG1		other	3	0.004	9	Insulin signaling
ERBB2		kinase	2.867	0.002	30	
MEF2C		transcription regulator	2.704	1.79E-12	17	Growth
MYOD1		transcription regulator	2.53	2.19E-09	14	Growth
TGFB1		growth factor	2.453	3.50E-07	20	Growth and inflammation
HNF1A		transcription regulator	2.439	1.90E-04	47	Cholesterol metabolism
IL1R1		transmembrane receptor	2.39	7.36E-03	22	Inflammation
SMAD3		transcription regulator	2.353	3.01E-05	6	Cell differentiation
Akt		group	2.311	1.08E-04	6	signaling of insulin and others receptors
IGFBP2		other	2.219	0.01	17	Growth
STAT5a/b		group	2.213	0.006	15	
SRF		transcription regulator	2.204	3.01E-07	6	Cell proliferation and differentiation
BDNF		growth factor	2.202	0.001	7	Growth
FSHR		G-protein coupled receptor	2.177	0.008	5	
RB1		transcription regulator	2.165	1.39E-04	5	
MAPK8		kinase	2.138	0.002	7	Regulation of development
ESR1		ligand-dependent nuclear receptor	2.093	1.20E-07	26	Growth
TBX5		transcription regulator	2.091	3.75E-08	11	Regulation of development
COL6A1	0.68	other	2.064	3.56E-06	6	Growth
MET	0.799	kinase	2.039	0.001	25	Development and organogenesis
PELP1		other	2	0.003	10	Inflammatory signaling

[1]Positive z-scores predict an overall increase in the activity of the regulator in Duroc pigs, while negative z-scores indicate a prediction of an overall increase in the regulator activity in Iberian.

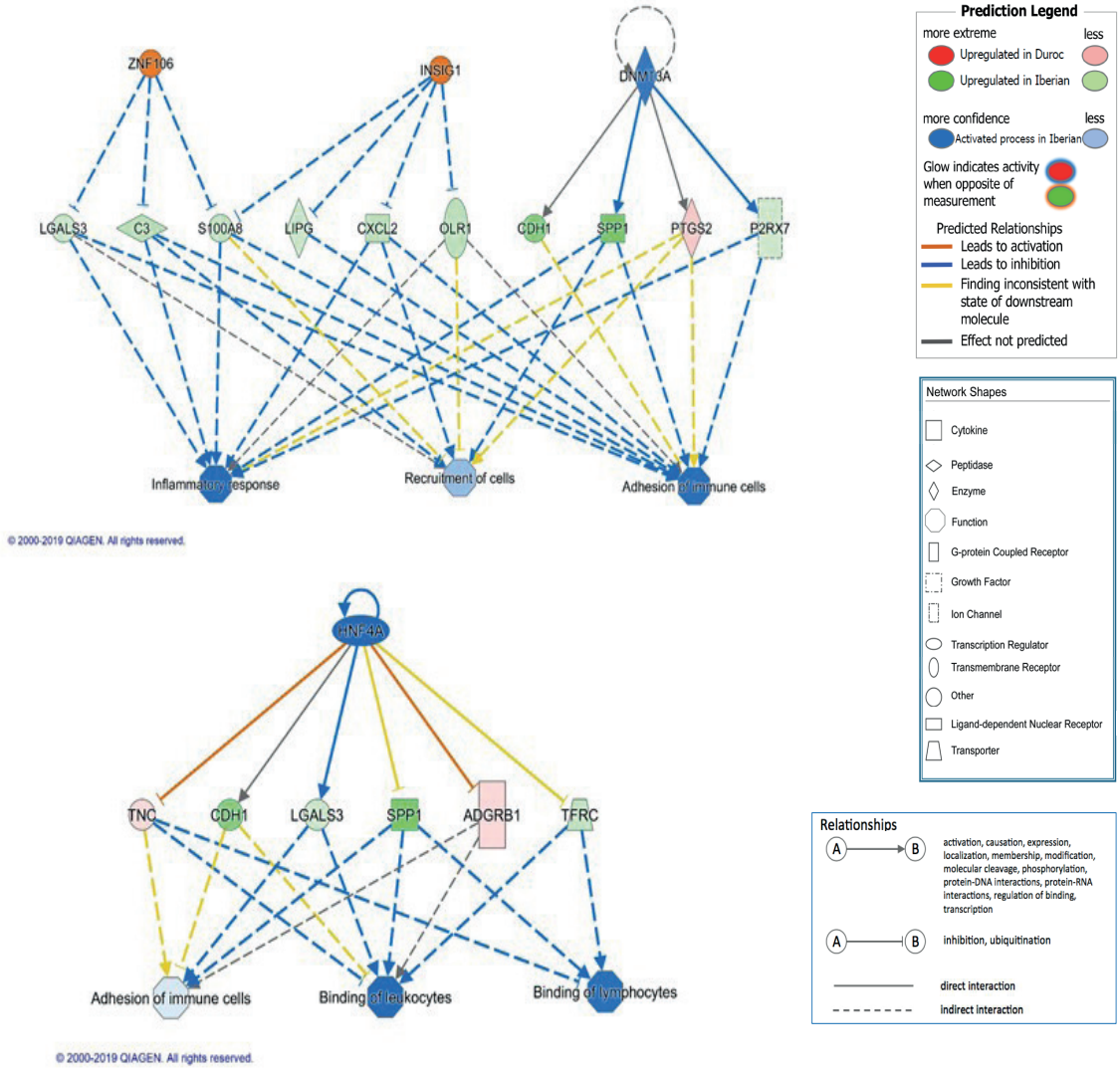
On the other hand, calcium signaling and actin cytoskeleton signaling pathways were activated in Duroc pigs. Calcium signaling influences signal transduction in cells, thereby activating cellular growth and development (Wilmann et al., 2000), and actin cytoskeleton signaling pathways play an important role in the organization of the cytoskeleton and in dynamic processes such as cell motility, vascular permeability, axon guidance, cytokinesis, and phagocytosis (Kanzaki et al., 2001). In agreement, enriched biological functions were mainly related to organismal development (i.e., size of animal, mass of organism, and size of body), and cellular assembly and organization (i.e., organization of cytoskeleton and cytoplasm). Significant activation (z-score > 2) was observed for functions related to growth (size of animal).

The upstream analysis and regulator effects tools of the IPA package were employed to identify potential transcriptional regulators that may explain the differential patterns of expression observed between breeds, and allowed the identification of 739 regulators (p-value < 0.05; Table S6 and Table 3).

Moreover, the sense of activation state was predicted for some of them. In Iberian pigs ten upstream regulators were activated (z-score < −2), mainly related to inflammatory response, such as KDM51, DNMT3A, AHR, NR1H3, NOS, or RBPJ, and lipid metabolism, such as AHR, NR1H3, or MED1. Especially NR1H3, also known as LXRA, is a transcription factor that modulates immune and inflammatory responses in macrophages and is a regulator of macrophage inflammatory signaling (Zelner and Tontonoz, 2006). In adipocytes, LXR may be constitutively active and it regulates glucose uptake, adipocyte differentiation, and adipogenesis (Laurencikiene & Ryden, 2012). Also, it is interesting to note that the two most significant regulators activated in Iberian pigs, the histone demethylase KDM5A and the methyltransferase DNMT3A, have known key roles in epigenetic regulation, including emerging roles in the epigenetic regulation of inflammation and immune functions (Kang et al., 2017).

In contrast, 23 upstream regulators related to cell proliferation, differentiation, and growth were activated in Duroc pigs (p-value < 0.01, z-score > 2, Table 3) as TGFB1, MEFC2, IGFBP2, SRF, or MYOD1, in agreement with the different development of the compared breeds. Transforming growth factor (TGF)-β represents a prototype of multifunctional cytokine (Han et al., 2012). The TGFB1 gene regulates various cell activities inside the cell, including the growth and

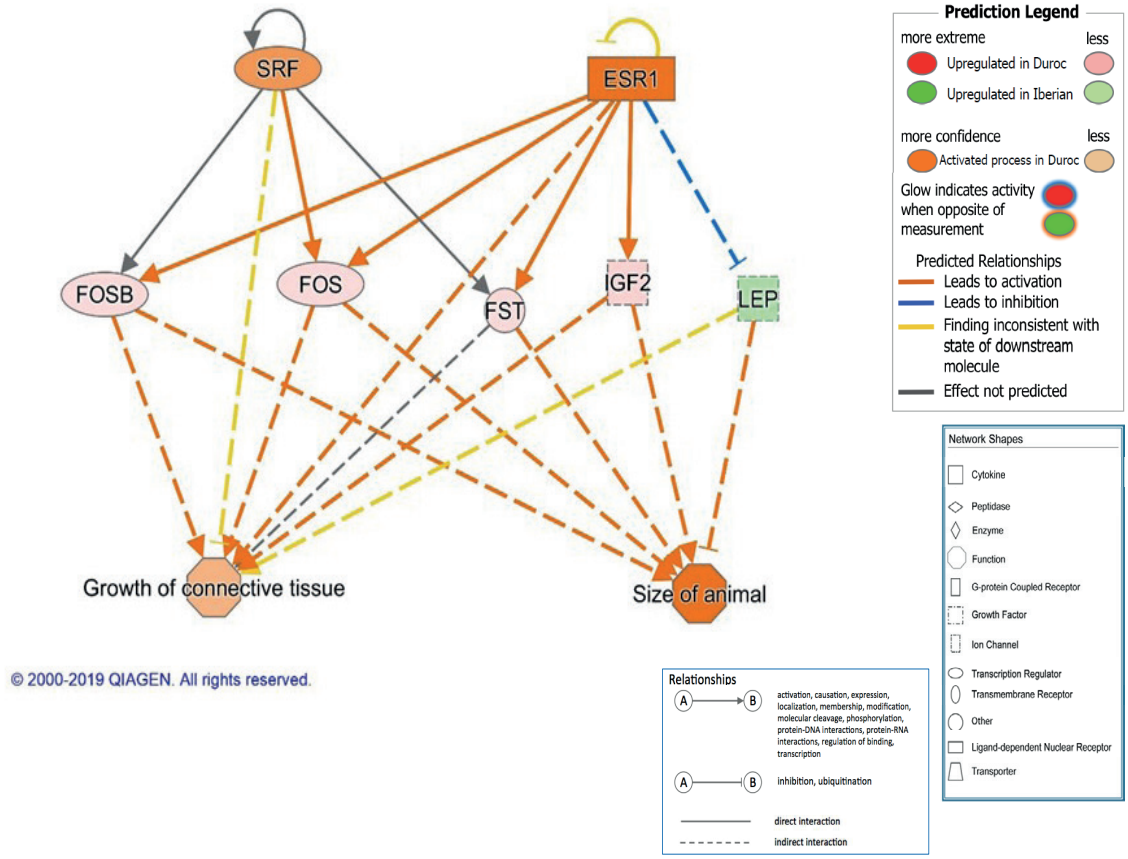
Figure 1. Regulator effects networks predicted as activated in Iberian pigs



division (proliferation) of cells, the maturation of cells to carry out specific functions (differentiation), cell movement (motility), and controlled cell death (apoptosis). Its broad activities include, among others, context-specific inhibition or stimulation of cell proliferation and control of extracellular matrix (Verrecchia and Mauviel et al., 2002). Insulin-like growth factor (IGF) signaling plays a pivotal role in cell proliferation and mitogenesis (Migita et al., 2010). IGFBP2 is a negative or positive regulator of cell adhesion, migration, and invasion, in an IGF-independent manner. In the same way, IGFBP2 positively or negatively regulates cell growth and survival (Migita et al., 2010).

Several regulator effect networks were also identified. Interestingly, two out of 15 predicted regulatory networks were involved in inflammatory response and

Figure 2. Regulator effects network predicted as activated in Duroc pigs



predicted to be activated in Iberian pigs. These two networks, shown in Figure 1, include the transcriptional regulators ZNF106, INSIG1, DNMT3A, and HNF4A, which are involved in biological functions related to inflammatory response, and reflect their potential regulatory mechanisms in the expression of several DE genes.

In addition, another interesting regulatory network was identified and predicted to be activated in Duroc pigs that was involved in the size of the animal and the growth of connective tissue, and included the transcriptional regulators SRF and ESR1 (Figure 2).

In pigs, AT is the main tissue for fat synthesis but it is also an endocrine organ that regulates the production of various hormones, growth factors, and cytokines in response to nutrient and hormonal signals. The enrichment of genes and functions involved in glucose and lipid metabolism in Iberian pigs may be indicative of a more intense employment of carbohydrates as fuel and for lipid synthesis and higher accumulation of AT, in agreement with their obese phenotype (Nieto et al., 2002; Fernandez-Figares et al., 2007 and Switonski et al., 2010). The increase

in AT mass in obesity is associated with profound histological and biochemical changes characteristic of inflammation (Fliers, 2004 and Weisberg et al., 2003). Several studies have shown that preponderance of pro-inflammatory versus anti-inflammatory immune cells is a hallmark of obesity-associated chronic low-grade inflammation, which leads to macrophage infiltration and accumulation in AT of obese animals (Gustafson et al., 2007 and Pahlvani et al., 2017). Our gene expression results indicate an increased inflammatory response in the Iberian AT, compatible with low-grade inflammation being developed as a consequence of the adipose tissue expansion and lipid accumulation characteristic of the breed. Also, upregulation of leptin in Iberian pig AT may contribute to recruitment of phagocytes, as it has been observed that leptin is a potent monocyte chemoattractant in in vitro studies (Gutierrez et al., 2009).

Accumulation of lipids and the consequent inflammation are also related to the release of a range of factors that predispose toward insulin resistance (Gutierrez et al., 2009 and Lee and Lee, 2014). In fact, Iberian pigs have been proposed as a model for obesity, leptin resistance, and insulin resistance (Torres-Rovira et al., 2011). In the present work, insulin (INS), its receptor (INSR), and insulin-induced 1 (INSIG1) signaling is predicted to be increased in Duroc pigs, with INSIG1 being significantly activated (z-score = 3), in agreement with a reduced insulin sensitivity in Iberian pigs. In obese mammals, accumulation of inflammatory cells has been associated with systemic hyperinsulinemia and insulin resistance, reducing insulin sensitivity locally in AT, as well as on insulin effects in other organs (Xu et al., 2003; Apovian et al., 2008 and Gutierrez et al., 2009). Also, we found overexpression of *GLUT4* in Iberian pigs. Increase in *GLUT4* expression selectively in fat enhances whole body insulin sensitivity and glucose tolerance, even in diabetic mice (Sheperd et al., 1993; Chung et al., 2010 and Stall et al., 2014). Thus, the *GLUT4* results may be indicative of an adaptive response to insulin resistance caused by the low-grade inflammation that occurs in these obese animals.

It is interesting to note that our results suggest metabolic alterations related to fattening, such as adipose tissue inflammation and insulin resistance, starting to develop in young growing Iberian animals. This early process may seem surprising, but there is evidence in humans demonstrating that the initiating events in obesity-induced inflammation can occur in all developmental stages,

including early in infancy, childhood, and adolescence (Singer and Lumeng; 2017). In fact, the loci that contribute genetic susceptibility to human obesity are known to play a dominant role in regulating weight and fat mass from the first years of life (Elks et al, 2014). In our animals, at this early developmental stage there is already a striking difference in the subcutaneous adipose tissue development between breeds (24.1 mm vs. 10.7 mm for back fat and 27.8 mm vs. 15.7 mm for ham fat thickness in Iberian and Duroc pigs, respectively; *p*-value < 0.001 (Benítez et al, 2018)). Moreover, besides the genetic predisposition, the development of inflammation may be exacerbated in our animals due to the low level of n-3 FA provided in the diets (1.5g kg^{-1}), which is normal in the cereal-based concentrates commonly used for growing pigs.

In contrast, in the Duroc breed we observed the enrichment of functional categories, pathways, and regulatory networks involved in the size of the animal or mass of the organism, both related to the GO category "organismal development". This result is associated with the overexpression in Duroc of several genes and regulators with a known role in development, such as *IGF2, MYCN, FMOD, FOSB, RGS7, AKT1, PHGBH*, or *FOS*, and is in accordance with the usual phenotypic growth differences between Iberian and Duroc breeds, as explained in previous work (Óvilo et al., 2014b and Ayuso et al., 2015). Interestingly, although the body weight of our experimental groups was similar, the tissue distribution was not. Iberian animals showed higher fat content in the carcass, while Duroc showed increased yield of premium cuts, which confirms the higher lean tissue development in the latter. In previous studies, the muscle transcriptome of Duroc crossbred pigs was characterized by activation of cellular and muscle growth pathways, in agreement with the present results (Ayuso et al., 2015). However, the comparison between breeds and genotypes had not been performed before at the level of the AT, where the net cell differentiation ability is expected to be higher in Iberian pigs. Then, the enrichment and activation of growth functions and pathways in Duroc pigs may indicate a coordinated regulation of such general developmental processes in the different tissues, in agreement with the known higher growth potential of Duroc pigs. It is also interesting to note that due to the greater appetite of the Iberian breed, the feed intake was higher in this breed, implying that feed conversion ratio was lower in Duroc than Iberian pigs, in agreement with their higher growth efficiency.

In Duroc pigs, several biological functions, pathways, and regulators involved in the organization of the cytoskeleton and the extracellular matrix (ECM) were enriched. In AT, adipocytes are embedded in the ECM, which provides structural support and anchorage for cells and is composed of the same proteins found in other tissue types (Mariman and Wang., 2010). The enrichment of ECM functions seems to be a consequence of the observed up-regulation in Duroc pigs of genes coding for structural proteins, such as myosins, tropomyosins, troponins, actins, collagens, laminins, and integrins, which in non-muscle cells are involved in regulating cytokinesis, cell motility, and cell morphology (Bresnick, 1999 and Shutova and Svitkina., 2018). Moreover, collagens may serve different purposes during early and late stages of adipocyte development (Mariman and Wang., 2010), and some myosins and actins, as well as the activation of actin cytoskeleton signaling pathways, have a role in glucose uptake in adipocytes, improving plasma membrane permeability and thus favoring insulin-stimulated glucose transport (Stall et al., 2014). In this process, calcium and calmodulin genes (such as *CAMK2A* and *CAMK2B*) are also implicated, regulating their co-localization at the plasma membrane of adipocytes (Stall et al., 2014). In agreement, *CAMK2A* gene (FC = 23,6) and other genes implicated in calcium signaling pathways, such as *CACNA1S, CACNG1, CANG6*, and *CACNB1*, were also upregulated in Duroc pigs.

The ECM composition is related to development stage, viability, and subtype of the adipocytes (Mariman and Wang., 2010). At early adipogenesis stages, during pre-adipocyte differentiation, the ECM is characterized by constructive processes, with an increase in many structural ECM components. In contrast, in mature adipocytes there is a balance between construction and degradation processes of ECM, known as ECM remodeling (Catalán et al., 2012). Thus, as adipogenesis progresses, the storage of fat in the adipocytes is paralleled by changes of the ECM. Our results agree with a denser ECM in Duroc AT (Horodyska et al., 2019), which may suggest a more precocious stage of AT development in these animals. Moreover, a dense ECM has been proposed to be related to reduced adipogenesis (Horodyska et al., 2019; Bouloumie et al, 2002). Also, it is important to keep in mind that insulin stimulates the formation of ECM (Wang et al., 2006), and thus, the insulin resistance in Iberian animals may have a role in limiting ECM development. At last, the hypertrophy of adipocytes in our obese Iberian animals

and the inflammatory status and a potential hypoxic stage may negatively influence the maintenance of the ECM. All these hypothesis may contribute to the observed activation of the factors involved in the construction of the ECM in Duroc breed or may lead to greater instability in the ECM of the Iberian animals. Nevertheless we cannot discard that this effect is due to the mixed nature of the tissue sample employed, which includes different cell types, and in which breed may account for a differential abundance of mature adipocytes versus preadipocytes and other cells. Also, we have to acknowledge that the characteristic and differential feed intake observed between the employed breeds may, in part, be involved in the transcriptome differences observed. In the experimental design employed in our study the breed effect cannot be separated from the intrinsic higher appetite of the Iberian pigs. For this purpose, another experimental design with restricted feeding could be employed in future works.

3.4.4.2. Diet Effects and Interaction between Breed and Diet on Transcriptome

Considering the strong effect of breed on transcriptome, and the fact that both breeds showed the quoted difference in feed intake, the effect of the diet was evaluated separately in the two breeds. DESeq2 and Cufflinks identified 49 and 207 DEGs according to diet in Iberian pigs, and 5 and 57 DEGs according to diet in Duroc pigs, respectively. Out of the DEGs affected by diet in Iberian pigs, 23 were common to both employed methods (Tables S7 and S8), while only three DEGs (*CYP1A1*, *ADGRF2*, and *TMEM182*) were common to both methods in Duroc pigs. Due to the scarce significant findings obtained with DESeq2 software, and taking into account that diet effects on gene expression detected in the Cufflinks pipeline were mostly successfully validated by qPCR, we decided to employ the Cufflinks output in order to be able to perform a functional interpretation of the diet effects on transcriptome. Thus, 207 DEGs affected by diet in Iberian pigs and 57 in Duroc pigs were employed for functional analysis. We have to consider that the performed intra-breed analysis of the diet effect implies that a lower biological replication was employed (6 vs. 6 animals in each comparison), meaning that the analysis may be considered less consistent than that of the breed effect and would benefit from additional analysis with a higher number of subjects, as employed in the validation step.

Table 4. IPA-based list of pathways in the set of DEGs according to diet in Iberian and Duroc pigs (*p*-value ≤ 0.01).

Canonical Pathways in Iberian	*p*-value	Ratio [1]	z-score [2]	Molecules
Complement System	2.45471E-08	7/37	-0.816	*C4A/C4B,C4BPB,C4BPA,C1QC,C-1QA,C1QB,CR2*
Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	2.0893E-05	3/7		*DLAT,DLD,PDHB*
Agranulocyte Adhesion and Diapedesis	0.00024	8/192		*MYH4,SELE,CXCL14,CCL24,C-CL14,CCL26,ACTA1,MYL1*
Mineralocorticoid Biosyn-thesis	0.0034	2/10		*EBP,HSD3B1*
Interferon Signaling	0.004	3/36		*MX1,IFI6,ISG15*
Glucocorticoid Biosynthesis	0.004	2/11		*EBP,HSD3B1*
Aldosterone Signaling in Epithelial Cells	0.004	6/174		*HSPA8,DNA-JB4,HSPH1,HSPA13,DNAJB1,D-NAJA1*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	0.005	6/186		*MYH4,IGFBP3,IL10RA,CD14,-SERPINE1,MYL1*
Androgen Biosynthesis	0.006	2/14		*EBP,HSD3B1*
Protein Ubiquitination Pathway	0.009	7/271		*HSPA8,DNA-JB4,HSPH1,HSPA13,PSMD14,D-NAJB1,DNAJA1*
Glucocorticoid Receptor Signaling	0.01	8/350		*HSPA8,SELE,KRT8,CDK7,CD-KN1A,PRKAA2,CD163,SERPINE1*
Canonical Pathways in Duroc	***p*-value**	**Ratio [1]**	**z-score [2]**	**Molecules**
Protein Kinase A Signaling	0.002	5/400		*PPP1R14C,CAMK2A,PYGM,TN-NI2,MYLK2*
nNOS Signaling in Neurons	0.005	2/47		*CAMK2A,CAPN3*
Calcium Signaling	0.01	3/206		*CAMK2A,TNNI2,MYH8*

[1]Ratio= number of DEGs in a pathway divided by the number of genes comprised in the same pathway.
[2]Positive z-scores predict an overall increase in the activity of the pathway in CH diet while negative z-scores indicate a prediction of an overall increase in activity in HO diet.

In Iberian pigs, 124 DEGs were upregulated in HO diet and 83 in CH diet (Table S7). Functional analysis indicated that main functional categories, pathways, and regulatory routes affected by these DEGs were related to inflammation, lipid metabolism, and fat tissue development. Eleven canonical pathways were significantly enriched in the dataset (*p*-value < 0.01; Table S9 and Table 4), with none of them being significantly activated or inhibited due to the diet. The most significant among them was the complement system (*p*-value = 2×10^{-8}), which is

an important contributor to the low-grade chronic inflammatory status associated with AT metabolic alterations in obesity and metabolic disorders (Vlaicu et al, 2016). This pathway showed a negative Z-score implying a trend for activation in HO diet. Also, agranulocyte adhesion and diapedesis and interferon signaling, both pathways being essential in the immune response, were significantly enriched (p-values = 0.0002 and 0.003, respectively). Several other relevant enriched pathways were related to FA metabolism (acetyl-CoA biosynthesis I) or corticoid metabolism, such as mineralocorticoid biosynthesis, glucocorticoid biosynthesis, aldosterone signaling, androgen biosynthesis, or glucocorticoid receptor signaling (p-value < 0.01).

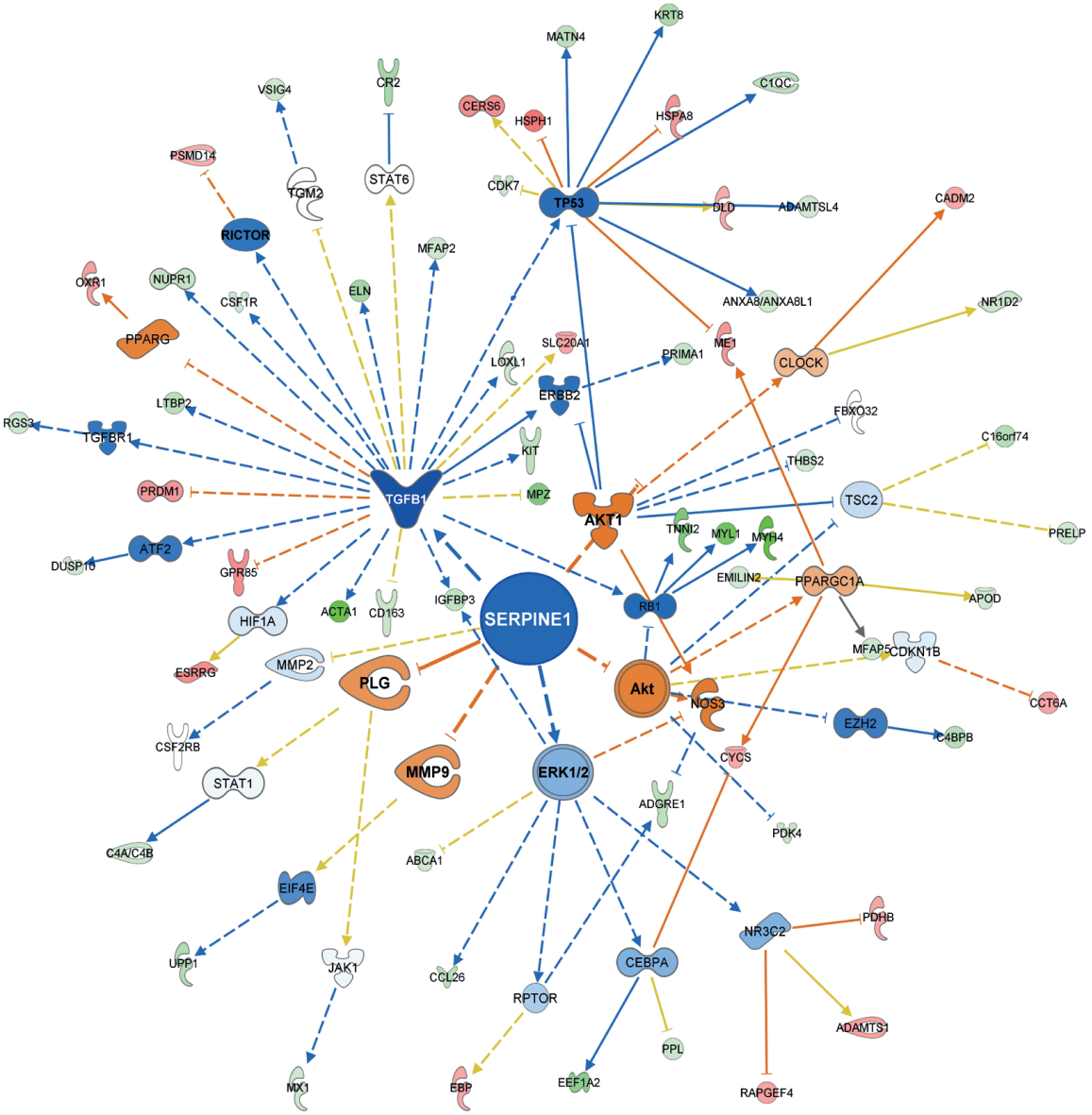
Corticoid metabolism is related to adiposity and inflammation (Marissal-Arvy et al., 2011) and corticoid levels have contradictory effects on lipid metabolism and immune response, but are usually considered to be associated with the complex phenotype of metabolic abnormalities in obesity and metabolic syndrome (Peckett et al, 2011 and Björntorp and Rosmond, 2000). For instance, chronic elevated levels of glucocorticoids are associated with lipogenesis, neoglucogenesis, adiposity, and obesity in humans (Boullu-Ciocca et al, 2005; Peckett et al, 2011), while acute GC increase is associated with lipolysis. Typically, GCs exert potent anti-inflammatory actions, yet chronic GC exposure has been linked to elevated inflammatory states (Johannsson et al., 2014). On the other hand, mineralocorticoids promote the expression of inflammatory cytokines in AT. The observed enrichment of corticoid biosynthesis is mainly due to DE of the HSD3B1 gene, which is upregulated in HO (2.33x) and catalyzes the oxidative conversion of hydroxysteroid precursors into ketosteroids, being crucial to the production of all classes of steroid hormones. Enrichment of corticoid biosynthesis and signaling may be indicative of diet effects on metabolic homeostasis, possibly mediating the maintenance of a balance of adequate plasma glucose levels and energy accumulation as triglycerides and influencing the development of chronic inflammation.

Regarding gene ontology enrichment, we detected that the enriched biological functions were involved in immune cell trafficking, inflammation, lipid, carbohydrate, and energy metabolism, and AT development (Table S10). Many functions related to recruitment, proliferation, expansion, movement, chemotaxis, and quantity of all the different immune cell types were affected by diet. Some

of these functions, especially those involved in recruitment, movement, and chemotaxis of myeloid cells and quantity of lymphocytes and monocytes, were increased in HO diet (negative z scores), while some other functions related to inflammation, binding, and quantity of myeloid cells were increased in CH group (positive z scores). These results are indicative of a low-grade inflammation being developed in AT with both dietary groups, with different processes and cell types being implicated in the response observed for each one. An obesity-associated, low-grade inflammation may have developed, as a consequence of the fat mass increase, in the Iberian pigs subjected to both dietary treatments, as previously explained in the comparison with Duroc breed, but development of inflammation could have occurred in an uncoupled way in both diet groups. For instance, the functional term inflammation of organ, involving 33 DEGs, seems to be increased in CH diet, while the term inflammatory response, involving another 24 DEGs, seems to be increased in HO diet. Attending to the specific functional terms and cell types observed we can speculate that a more intense or earlier inflammatory response may be developing in HO in comparison to CH. Also, a different balance between pro-inflammatory and anti-inflammatory cells and functions may be suggested; for instance, phagocytosis by macrophages seems to be activated in HO, which could play a role in repair and tissue remodeling (Gordon and Taylor, 2005). The different pace or consequences of the immune responses between diets might be related to the differential effect of the dietary nutrients and present in the circulation (fat in HO vs. glucose in CH), as well as the specific FA types being accumulated in the AT of both dietary groups (MUFA in HO vs. SFA in CH diet), which are known to differentially influence the development of obesity-induced inflammation (Teng et al., 2014) Moreover, the sampling moment chosen in the present work may be too early to understand changes in inflammatory processes between diets, as the chronic inflammation requires a long period of fat accumulation before it becomes clearly discernible (Lee and Lee, 2014) and the inflammatory cell states change during the time course of the inflammation in a continuum range of functional states without clear boundaries, making it difficult to precisely infer the evolution of the inflammation process (Murray et al., 2014).

Also, functional categories related to lipid and glucose metabolism and AT development were affected by the DEGs, including terms such as quantity of adipose tissue, mass of fat pad, insulin resistance, oxidation of long chain fatty

Figure 3. Predicted causal network for the DEG and master regulator *SERPINE1*.



acid, fatty acid metabolism, energy homeostasis, quantity of carbohydrate, quantity of glycogen, or synthesis of lipid (Table S10). Results agree with higher levels of carbohydrates in the diet of CH group and its utilization for lipid synthesis, higher metabolism and oxidation of FA in HO group, and accumulation of AT in both groups.

The observed results regarding inflammation are a consequence of several chemokines (*CCL14*, *CCL24*, *CCL26*), and pro-inflammatory proteins (*SERPINE1*, *CD14*, *CR2*, *CSF1R*, *CSF2RB*), which were DE. For instance, *SERPINE1* gene was 1.61-fold upregulated in HO. It is a pro-coagulant protein, expression of which is increased in AT of obese animals and is used as a marker of AT inflammation (Weisberg et al., 2003). Besides the DE observed for this gene, the functional analysis pointed out *SERPINE1* as one master regulator for the expression differences obtained, and a causal network was constructed that relates this gene to other 30 regulators and 62 DE genes (Figure 3).

Also, *CSF1R* gene (FC = 1.57) is the receptor for colony stimulating factor 1, a cytokine which controls the production, differentiation, and function of macrophages (Sehgal et al., 2018). Several studies in obese mice comparing different diets enriched in SFA and MUFA indicated an increase of markers of inflammation and recruitment of macrophages, such as CSF1R, in SFA/MUFA diet (Todoric et al., 2006 and Han et al., 2010). These findings are in accordance with our results, as the HO diet has higher SFA and MUFA content than CH diet, with similar PUFA.

Also, some works demonstrated that inflammasome activation depends on reactive oxygen species (ROS), another inflammation-associated group of compounds, which are produced extensively by the activated phagocytes (Jo et al., 2016). Circulating dietary factors such as fatty acids, especially SFA, can increase the generation of ROS, in addition to triggering inflammatory signaling (Forrester et al., 2018). Accordingly, in our study, in Iberian obese pigs the generation of *ROS* biological function was also activated in HO diet.

In relation to FA metabolism biological functions activated in HO diet, the up-regulated genes were mainly related to lipid and cholesterol metabolism, glycolysis, and gluconeogenesis, such as *CYP1A1* or *PDK4. CYP1A1*gene encodes a member of the cytochrome P450 superfamily of monooxygenases, which convert saturated and unsaturated FAs into small molecules (Sridhar et al., 2017). PDK4

provides pyruvate and other three-carbon compounds for glucose synthesis when glucose is in demand. PDK4 up-regulation, beneficial in white AT in obese and insulin resistant rodents, leads to increased glyceroneogenesis and takes advantage of fatty acids as an energy source (Zhang et al., 2014). Thus, up-regulation of fatty acid metabolism genes in HO-fed Iberian pigs may indicate the employment of dietary fatty acids as an energy source.

The upstream analysis allowed the identification of 687 (p-value < 0.05) upstream regulators in Iberian pigs depending on diet (Table S11), and the sense of activation state was predicted for eight of them. Three upstream regulators were activated (z-score ≥ 2, p-value < 0.05) in CH diet related to cell differentiation, proliferation, and development, such as MAPK1. In HO diet, 5 upstream regulators were significantly activated (z-score ≤ −2, p-value < 0.05), some of them with roles in immune and inflammatory response, such as TGFB1 or IFNL1 (Han et al., 2012 and Meager and Das, 2005). The CCAAT/enhancer-binding protein β (CEBPB) is a transcription factor with classical functions in transcriptional and translational regulation of lipid metabolism, but it also has as a regulator role on differentiation and inflammatory processes, mainly in AT and liver (Rahman et al., 2007). In fact, it is considered a crucial transcriptional regulator of diet-induced inflammation and hyperlipidemia development (Rahman et al., 2016). Even with a low statistical significance (z-score = −1.34), our results showed activation of this regulator in HO diet. Thus, in general, functions, pathways, and regulators involved in inflammation are activated in HO and this may indicate that high circulating FA levels, coming from the diet in HO-fed Iberian animals, are more relevant in AT inflammation than the SFA accumulated within the adipocytes of CH group following de novo synthesis from the dietary carbohydrates.

In Duroc pigs, 57 genes were affected by diet (Table S8), with 47 showing higher expression in HO group and only 10 in CH group. Functional analyses yielded three enriched pathways (p-value < 0.01): protein kinase A signaling, nNos signaling in neurons, and calcium signaling (Table S9). In addition, we observed an increase of leukocyte migration biological function in the CH diet (z-score = 2.17, p < 0.05). Other enriched functions were mainly related to immune cell trafficking (i.e., recruitment of leukocytes, migration of phagocytes, and cell movement of leukocytes), lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism (i.e., concentration of lipid, conversion

and metabolism of retinol, and modification of retinaldehyde) (Table S10). The upstream analysis allowed the identification of 169 potential regulators for the diet effect in Duroc pigs (p-value < 0.05), with none of them being significantly activated or inhibited (Table S11). Thus, functional interpretation of DEGs in Duroc pigs is less clear than in Iberian pigs. Although some functional categories coincide, the significance is reduced and there is a lack of consistent activation or inhibition of pathways or functions. Moreover, it is interesting to note that a few biological functions related to immune cell trafficking, such as recruitment of leukocytes or leukocyte migration, showed an opposite response to diet in both breeds, being activated in HO-fed Iberian pigs but not in CH-fed Duroc pigs.

Out of 207 DEGs affected by diet in Iberian pigs (Table S7) and 57 in Duroc pigs (Table S8), only seven DEGs were common, which were affected by diet in both breeds. The range of FC was larger in the Iberian pigs (FC from 4.65 to 21.5) than in Duroc pigs (FC from 6.26 to 6.86). Thus, results indicate a more intense transcriptomic response to diet in Iberian than in Duroc pigs, in terms of both the number of DEGs as well as the magnitude of the effects. Additionally, one out of the seven common genes affected by diet in both breeds showed an opposite regulation in Iberian and Duroc pigs—*SERPINE1* gene was up-regulated in HO diet in Iberian and in CH diet in Duroc pigs. These results are indicative of an interaction between the diet and the breed effects.

In order to deepen the potential interaction effects unraveled from Cufflinks outputs, we used DESeq2 tool to perform a joint analysis using a full model fitting the breed–diet interaction. This analysis yielded three genes with significant interaction effects (FDR < 0.05): *JAZF1* and two novel genes located in chromosomes 15 and 13, with strong homologies to human *TTN* and *XIRP1* genes, respectively. Juxtaposed with another zinc finger, gene 1 (*JAZF1*) is a newly identified gene that is associated with various types of cancer and diabetes mellitus (Thomas et al., 2008; Zeggini et al., 2008). Recently, studies have also shown that the *JAZF1* gene reduced lipid synthesis and increased lipolysis by regulating the level of expression of genes related to fat metabolism in mice (Li et al., 2011) and because it decreases the maturation of lipid droplets and fat storage (Jang et al., 2014). Therefore, this gene plays a critical role in the regulation of lipid homeostasis and is considered a candidate to affect meat quality (Yang et al.,

2015).The RNA-Seq results obtained for this gene, which showed a qualitative interaction, were validated by qPCR (Table 1).

Interestingly, the gene showing opposite diet effects in the two breeds (*SERPINE1*), which is probably one of the most extreme cases of qualitative interaction, does not have a significant interaction *q*-value according to DESeq2 output. In fact, two genes showed significant nominal *p*-values in the interaction output (*p*-values = 0.001 and 0.005) but did not reach the FDR threshold, namely *SERPINE1* and *CYP1A1*, and were included in the qPCR validation step. Significant interaction effects were technically and biologically validated for these two genes with the more precise qPCR quantification and with a wider sampling (all 48 available animals instead of the 24 employed for RNA-Seq). These findings suggest that available statistical tools for analyzing interaction effects are prone to false negative results, or the multiple testing correction is too demanding. Also, we have to consider that the magnitude of the expression changes which leads to biological consequences is unknown. Taking these considerations into account, we tried a less demanding threshold to employ a list of genes with suggestive interaction effects for functional interpretation. We considered the upper part of the list of genes obtained from DESeq2 output, ordered by significance, assuming as the threshold the nominal significance of *CYP1A1* gene interaction effect (nominal *p*-value ≤ 0.005). This list includes 49 transcripts corresponding to 44 known genes (Table S12). Approximately half of these genes show opposite response to diet in both breeds (23) and most quantitative interactions were detected for genes responding to diet in Iberian pigs but not in Duroc pigs. The functional analysis of this subset of genes showed significant enrichment of functions involved in inflammatory response and cellular movement, mainly affecting immune cells (*p*-values ranging from 9×10^{-3} to 3×10^{-5}) and organismal survival or death (*p*-values ranging from 2×10^{-3} to 3×10^{-6}), with half of the analyzed molecules being involved in these functions. Also, the network analysis showed that these genes were involved in metabolic disease, tissue abnormalities, and cellular assembly and organization. Although interaction effects are not clear and are difficult to interpret, joint results may agree with the differential fattening of the two breeds, leading to a clear inflammatory response being observed in Iberian pigs, which in turn is being developed with a different paucity in the two dietary groups. The between-breeds opposite regulation that diet exerts on

the expression of some genes is intriguing and warrants further research with longitudinal time-series experiments and a more comprehensive study of tissue development and inflammation. Breed–diet interactions have been unveiled at the phenotypic level (Olivares et al, 2009; Wood et al, 2004; Godinho et al, 2018) and at the candidate gene expression level (Zhao et al, 2004; Chung et al, 2007;Benítez et al., 2018). Nevertheless, to the best of our knowledge this is the first transcriptome study evaluating this kind of interaction effects in a domestic animal species. Our results highlight both the scientific interest and the technical difficulties intrinsic to this approach.

3.4.5. CONCLUSIONS

We found a deep effect of breed on adipose tissue transcriptome. Most differences in transcriptome between Iberian and Duroc breeds were related to growth, extracellular matrix formation, lipid and carbohydrate metabolism, and inflammatory and immune response. Results suggest low-grade inflammation and insulin resistance being developed in the obese Iberian animals in spite of the young age of the animals.

On the other hand, diet and interaction effects indicate a more intense response to diet in Iberian pigs, which is essentially translated into changes in genes involved in inflammation, immune response, lipid metabolism, and fattening. The higher transcriptional response observed in Iberian pigs may be associated with structural variants increasing sensitivity of gene promoters to nutrient stimulation, for instance affecting glucose response elements located within regulatory regions. Future studies should address the structural variation in these genomic regions, as well as the expression of long non-coding RNAs, which could provide insight into other regulatory mechanisms supporting the breed and diet influences on transcriptome. More extensive research on the basis of breed and diet effects, as well as diet-breed interactions, may contribute to deepening the understanding of adipocyte cell biology, lipid, and glucose metabolism differences between this obese Iberian phenotype and lean genetic types.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4425/10/8/589/s1. Table S1: Diet composition, Table S2: Primer design for qPCR validation, Table S3: Differentially expressed genes for the breed effect, Table S4: Canonical pathways enriched in the set of differentially

expressed genes affected by breed, Table S5: Biological functions enriched in the set of differentially expressed genes affected by breed, Table S6: Regulators predicted for the set of differentially expressed genes affected by breed, Table S7: Differentially expressed genes for the diet effect in Iberian breed, Table S8: Differentially expressed genes for the diet effect in Duroc breed, Table S9: Canonical pathways enriched in the sets of differentially expressed genes affected by diet, Table S10: Biological functions enriched in the sets of differentially expressed genes affected by diet, Table S11: Regulators predicted for the sets of differentially expressed genes affected by diet, Table S12: DESeq2 results for the interaction breed–diet effect.

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4. DISCUSIÓN GENERAL

4.1. EL CERDO IBÉRICO Y LA NUTRIGENÓMICA

En la actualidad, la mayor parte de la producción cárnica porcina corresponde a razas muy seleccionadas que poseen un alto potencial de crecimiento y que producen eficientemente carnes magras. Sin embargo, en ciertos países de la franja Mediterránea, como es el caso de España, este tipo de producción coexiste con razas autóctonas no seleccionadas, con crecimiento poco eficiente pero con una mayor capacidad de acumulación grasa y mayor calidad de carne. Este es el caso de la raza ibérica, una raza local caracterizada por un crecimiento lento, un escaso desarrollo muscular y una gran capacidad de acumulación de grasa, que además posee una gran adaptación al medio y rusticidad. Su producción es un claro ejemplo de sistema productivo sostenible orientado a la obtención de productos cárnicos de alta calidad, en el que, además de los aspectos genéticos, los aspectos ambientales y de manejo (especialmente los nutricionales) tienen una influencia decisiva en la composición tisular y son determinantes en la calidad de los productos. Su sistema tradicional de producción consiste en la restricción de alimento hasta el periodo final del engorde o montanera, que está basado en la ingesta de bellotas (ricas en ácido oleico) y pasto, y que conduce a un cambio en el perfil lipídico del músculo y de la grasa, caracterizado por un aumento en el contenido de AGMI, especialmente de ácido oleico (López-Bote, 1998). Este perfil confiere unas características organolépticas y tecnológicas especiales a su carne e influyen notablemente en la calidad excelente de sus productos curados. Estas peculiares características de la raza ibérica, unidas a sus diferentes sistemas de producción (montanera, cebo de campo e intensivo), hacen que sea una raza en la que las interacciones genética-nutrición tengan un potencial papel relevante en el fenotipo, lo que hace del cerdo ibérico un material animal idóneo para los ensayos nutrigenómicos.

En general, la nutrición y la genética han sido abordadas como disciplinas separadas ignorando las interacciones genoma-nutrición y sus posibles consecuencias sobre el metabolismo, fisiología y fenotipo del animal. El uso de herramientas multidisciplinares, como la nutrigenómica, y de nuevas herramientas moleculares como la secuenciación masiva del transcriptoma ofrece nuevas oportunidades para investigar estas interacciones complejas y nos aportan información muy valiosa y completa, que nos permite analizar de una manera global los procesos que ocurren en un determinado tejido y ante distintos estímulos. El verdadero

reto consiste precisamente en interpretar e integrar la enorme cantidad de información generada y extraer de ella información biológica relevante.

Las dietas suplementadas con AG, en especial con ácido oleico, se han venido empleando en los sistemas de campo y cebo intensivo de porcino ibérico, para conseguir una monoinsaturación de los tejidos que mimetiza el sistema tradicional de engorde en montanera. La modificación de la composición de AG de los tejidos puede deberse a la acumulación directa de los componentes de la dieta, pero también a la modulación de la síntesis endógena mediante cambios en la expresión génica y en la actividad enzimática (Diaz-Rúa *et al*., 2015; Reynés *et al*., 2017), debido a los efectos bioactivos de los nutrientes. Estos efectos pueden afectar a genes involucrados en la regulación de la adipogénesis, la lipogenésis y la lipolisis, modificando así la cantidad y composición de AG de los distintos tejidos y por tanto su calidad. Pero no es solo importante modular la composición de los tejidos para influir en su calidad a nivel sensorial y tecnológico, sino que controlar esa composición también puede influir de modo relevante sobre importantes aspectos nutricionales de los alimentos relacionados con la salud de los consumidores. El empleo de dietas enriquecidas en distintos tipos de AG y su efecto puede estudiarse por tanto desde un enfoque nutrigenómico, investigando los efectos que los AG de la dieta tienen sobre el fenotipo y que están vehiculados por cambios en la expresión génica (Corella y Ordovas, 2009). Este tipo de ensayos pueden realizarse con distintas aproximaciones. La más tradicional es la basada en hipótesis o abordaje de genes candidato, consistente en el estudio de la expresión de un panel de genes relacionado con un proceso o procesos de interés, habitualmente mediante PCR cuantitativa en tiempo real. El segundo tipo de abordaje, libre de hipótesis, consiste en el estudio global (omico) de la expresión del transcriptoma de un determinado tejido. Para esta segunda estrategia en los últimos años se está empleando la tecnología de secuenciación masiva del ARN, o RNAseq, aunque también pueden emplearse microarrays de expresión. El objetivo general de los estudios de expresión diferencial consiste en identificar los genes cuya expresión ha cambiado significativamente entre dos condiciones diferentes.

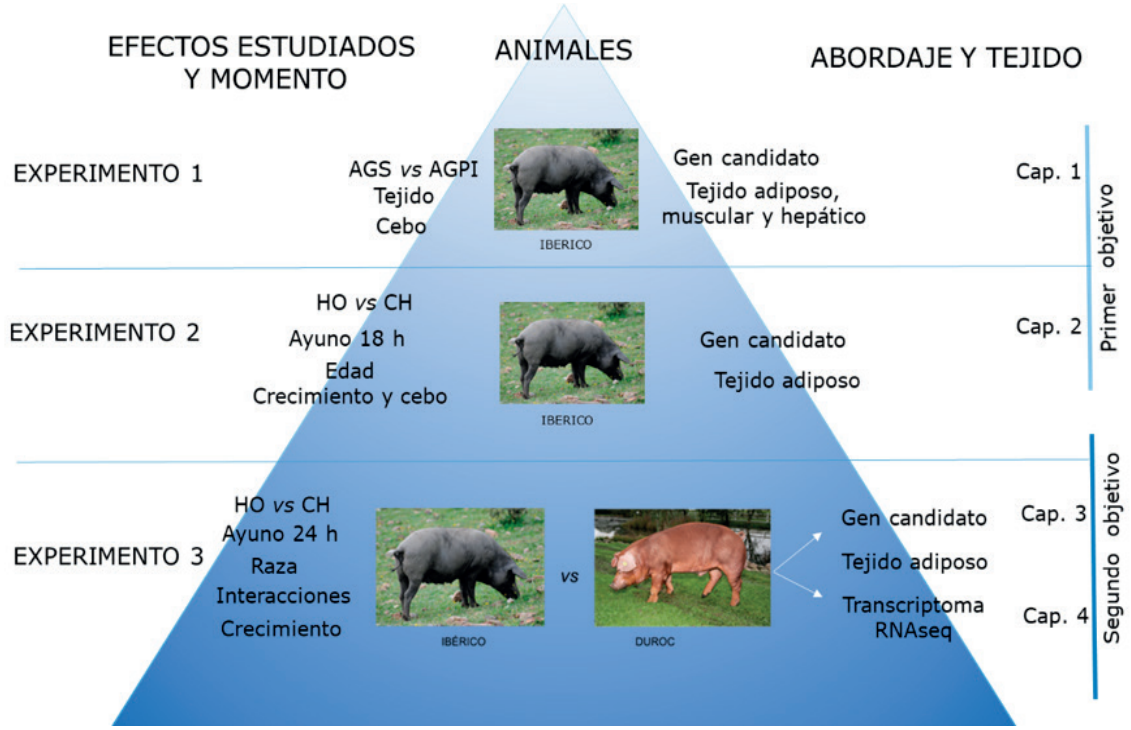
Ambas estrategias tienen ventajas e inconvenientes. Los estudios de transcriptoma nos aportan una información mucho más completa que el abordaje de gen candidato, ya que nos permiten capturar todos los tránscritos expresados

cuantificando su expresión, pero también ARNs no codificantes y mutaciones post transcripcionales (Wang *et al*., 2009). Estas aproximaciones permiten el análisis global de todos los genes que están participando en una determinada ruta metabólica, no limitándose a un número determinado de genes escogidos previamente con mayor o menor acierto. Además, nos permiten investigar no sólo los cambios en la expresión génica, sino también cambios de secuencia en el ARN mensajero como SNPs y otras variantes estructurales (Qian *et al*., 2014). Los principales inconvenientes de los estudios transcriptómicos son su elevado coste y la complejidad del análisis bioinformático de los datos, debido a los enormes volúmenes de información generados. En la práctica, el elevado coste conduce a tamaños muestrales reducidos y la multiplicidad de tests deriva en umbrales de significación exigentes. Todo esto se traduce en la detección de genes con diferencias de expresión de magnitud considerable, pudiendo estar limitada la detección de genes biológicamente relevantes, cuyas diferencias de expresión sean más pequeñas. La estrategia de gen candidato tiene una implementación más sencilla y menos costosa, pero requiere información a priori de estructura, función y expresión de los genes elegidos, que en muchos casos suele ser limitada o escasa (Li, 2013). Ambos abordajes son complementarios y según los objetivos es frecuente su uso combinado. Por ejemplo, la PCR cuantitativa es necesaria para la validación técnica y biológica de los resultados transcriptómicos, permite una cuantificación más precisa y por su menor coste puede aplicarse con mayor replicación biológica.

Por otra parte, ambos abordajes tienen limitaciones comunes a todos los estudios de expresión génica, principalmente referido a los aspectos de la variabilidad en la expresión dependiente de tiempo y espacio (tejido y momento del muestreo), pues la expresión génica es muy variable entre tejidos (incluso entre distintas localizaciones en un mismo tejido) y entre los distintos estadios en el desarrollo del animal (GTEx Consortium, 2017; Ferraz *et al*., 2008).

Los estudios nutrigenómicos tienen una orientación de investigación básica, pues permiten estudiar la respuesta biológica de los tejidos a un determinado factor, y relacionarla con los datos fenotípicos, permitiendo una comprensión más profunda del metabolismo. Además, el estudio de la relación existente entre el empleo de intervenciones nutricionales y aspectos de composición tisular o calidad de carne tiene un interés inminentemente práctico en el ámbito de la produc-

Figura 13. Ensayos nutrigenómicos y transcriptómicos comprendidos en esta tesis



ción porcina. En esta tesis se han combinado los dos abordajes mencionados en ensayos de tipo nutrigenómico para estudiar el efecto de la suplementación de la dieta utilizando distintas fuentes de energía, el efecto diferencial entre razas y el efecto del estado de alimentación (ayuno / postprandial) sobre el metabolismo. Para ello se han realizado tres experimentos que han dado lugar a cuatro trabajos recogidos en los capítulos 1 a 4.

4.2. ESTUDIO DE MECANISMOS MOLECULARES IMPLICADOS EN LA REGULACIÓN DEL METABOLISMO LIPÍDICO Y EN EL EFECTO DE DISTINTAS FUENTES DE ENERGÍA DE LA DIETA EN EL CERDO IBÉRICO.

Para llevar a cabo el primer objetivo de la tesis se utilizaron dos ensayos distintos, llevados a cabo en cerdos ibéricos de la línea Torbiscal, con manejo de campo y con un abordaje de gen candidato.

En un primer ensayo se planeó realizar una exploración inicial de los posibles efectos de distintas fuentes de grasa sobre aspectos productivos de composición tisular y sobre el metabolismo lipídico de varios tejidos en cerdo ibérico. Para ello,

el diseño experimental original de este ensayo incluía tres grupos experimentales de animales, suplementados respectivamente con grasa saturada, monoinsaturada y poliinsaturada durante el periodo de cebo. Lamentablemente, el grupo suplementado con AGMI tuvo que ser descartado, después de completarse el periodo experimental, al detectarse un problema en la composición de dicho pienso. En consecuencia, la comparación entre dietas se limitó al contraste entre grasa saturada y poliinsaturada, en cualquier caso interesante tanto desde el punto de vista productivo como de salud del consumidor. En medicina humana, el alto consumo de AGS se relaciona con la obesidad y con el aumento de los niveles de colesterol en sangre, estando todo ello claramente vinculado al desarrollo de enfermedades cardiovasculares (Katan *et al*., 1994). Por el contrario, la sustitución de los AGS por AGMI o AGPI en la dieta disminuye el riesgo a ese tipo de enfermedades y podría estar implicado en su prevención (Wood *et al*., 2003; de Longeril & Salen *et al*., 2012). En este contexto, actualmente existe un número creciente de consumidores que prefieren carnes más magras y que en su composición presenten ratios más altos de AGMI y AGPI en relación a los AGS.

El efecto de la dieta sobre la composición tisular y la expresión génica se analizó en tres tejidos distintos (tejido adiposo, muscular esquelético y cardiaco, y hepático) permitiendo evaluar los aspectos diferenciales del efecto de la intervención nutricional dependientes del tejido. Para ello se analizó un panel de seis genes con funciones relevantes en los procesos de lipogénesis y metabolismo lipídico (*SCD*, *FASN*, *ME1* y *ACACA*). Además se estudiaron dos genes (*CPT1* y *HADH*) implicados en la β-oxidación en dos tipos diferentes de músculo, el cardiaco y el *longissimus dorsi*.

En paralelo al primer trabajo de esta tesis, nuestro grupo llevó a cabo otro estudio previo evaluando los efectos de la fuente de energía de la dieta (Óvilo *et al*., 2014a), comparando los efectos de dietas isoenergéticas enriquecidas con ácido oleico y carbohidratos. El análisis de composición titular y expresión génica se realizó a tiempo final (110 kg (SD=3kg) de peso vivo) observándose escasas diferencias y de pequeña magnitud en la expresión génica, sugiriendo una moderada respuesta biológica a este tipo de intervenciones nutricionales de larga duración. En este trabajo previo, los efectos de la dieta sobre la composición tisular se establecieron muy temprano después del comienzo del tratamiento, lo que sugirió que los efectos de la dieta sobre el metabolismo podrían variar en su

respuesta e intensidad a lo largo del tiempo, por lo que el momento de muestreo podría ser decisivo a la hora de obtener una mayor o menor respuesta.

Utilizando muestras de los animales experimentales empleados por Óvilo *et al*. (2014a) y teniendo en cuenta los resultados obtenidos en éste y en el primer ensayo con la suplementación con AGS *vs* AGPI, se planteó el segundo trabajo de esta tesis. Este se realizó en un solo tejido (tejido adiposo subcutáneo del jamón) en el que habíamos observado una mayor respuesta a la dieta de la expresión génica. Se estudió la intervención nutricional con dietas con diferente fuente de energía (ácido oleico *vs* carbohidratos) a corto y largo plazo. En este trabajo se empleó un panel más amplio de genes candidatos para el estudio de expresión diferencial. Los resultados se interpretaron empleando los datos de composición titular que habían sido publicados previamente (Óvilo *et al*., 2014a). Asimismo se evaluaron los cambios de expresión génica entre muestras tomadas a los mismos animales después de un ayuno de 18 horas y 3 horas después de la ingesta de alimentos, con el propósito de profundizar en el conocimiento de la regulación de la lipogénesis.

4.2.1. Efectos de la suplementación con AG sobre la composición tisular en cerdo ibérico

En ambos experimentos se estudió el perfil de AG en tres tejidos diferentes: grasa dorsal subcutánea (capas interna y externa), músculo (*longissimus*) e hígado.

En las dos capas estudiadas del tejido adiposo el contenido en AG reflejó en gran medida la composición de la dieta. Los principales efectos se observaron para los contenidos de AGS, AGMI y AGPI. En el primer trabajo se observaron niveles más elevados de AGMI y AGS en el grupo que recibió la dieta enriquecida en AGS (grupo S) y niveles más elevados de AGPI en el grupo que recibió la dieta enriquecida en AGPI (grupo P), en concordancia con la deposición directa de los nutrientes. Por el contrario en los tejidos muscular y hepático los efectos de la dieta fueron algo menos claros y con menor consistencia entre las fracciones lipídicas analizadas (lípidos neutros y polares). De hecho en estos tejidos (muscular y hepático) se observaron algunas interacciones significativas dieta*fracción ($p<0.0001$), incluso de carácter cualitativo. A diferencia del tejido adiposo, estos dos tejidos no mostraron efecto de la dieta sobre el contenido en AGS. En los tres

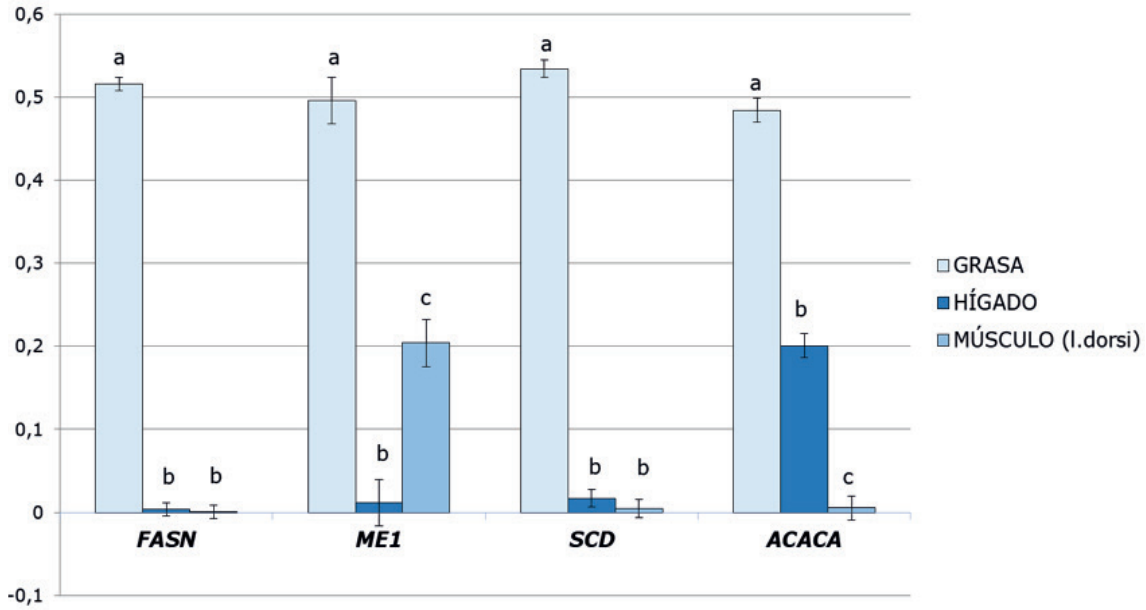
tejidos analizados la relación AGMI / AGS fue más alta en el grupo S y la relación AGPI / AGS fue mayor en el grupo P. El índice de desaturación C18:1n-9 / C18:0, que se considera una medida indirecta de la actividad de la enzima desaturasa o SCD (Attie *et al*., 2002), fue significativamente mayor en el grupo S. La principal conclusión es por tanto que la composición de AG de la dieta se refleja principalmente en el tejido adiposo y en menor medida en el músculo y el hígado. La falta de un contenido significativamente mayor de AGS en el músculo y el hígado en el grupo S, junto con el índice de desaturación más alto observado en las muestras de este grupo, indica que los AGS recibidos en la dieta se utilizan para la síntesis endógena de AGMI en los tejidos. De un modo similar, en el trabajo anterior (Óvilo *et al*., 2014a) observamos como los carbohidratos de la dieta se emplearon para síntesis de grasa saturada en los tejidos. Analizando conjuntamente los datos de composición de ambos trabajos se pone de manifiesto como los glúcidos estimulan la síntesis endógena de grasa saturada y los AGS estimulan la síntesis de AGMI, sugiriendo por tanto un papel activador de las dietas CH y AGS sobre la lipogénesis. A su vez la mayor deposición directa corresponde a los AGMI y AGPI.

4.2.2. Efectos de la suplementación con AG sobre la expresión génica en cerdo ibérico

En la comparación de dietas AGS *vs* AGPI, los genes lipogénicos analizados tuvieron una expresión muy variable dependiendo del tejido, con la mayor expresión observada en la grasa dorsal subcutánea, seguida del hígado y el músculo (Figura 14), excepto en el caso del gen *ME1* para el que la menor expresión se observo en el hígado.

La principal respuesta transcripcional a la dieta fue un aumento en la expresión hepática de los genes lipogenicos (*SCD*, *ME1* y *ACACA*) en el grupo S. En tejido adiposo se observó también un incremento de la expresión del gen *SCD* en el grupo S con significación sugestiva. Estos cambios en la expresión génica fueron consistentes con los efectos observados a nivel de composición tisular, con mayor contenido en AGMI y unos índices de desaturación mayores en este grupo, proporcionando una evidencia clara de la mayor lipogenesis y desaturación inducida por esta dieta. Nuestros resultados coincidieron con estudios previos que indicaron que los AGPI en la dieta, en su mayoría esenciales, aumentan el contenido de estos en los tejidos por deposición directa y se asocian con una menor

Figura 14. Nivel de expresión de los genes candidato según el tejido analizado. Barras con diferente letra indican diferencias significativas con $p < 0.0001$.



expresión de enzimas lipogénicas, mientras que una dieta rica en AGS induce la lipogénesis (Corominas *et al*., 2013; Dentin *et al*., 2005; Sampath *et al*., 2007). En particular, está descrito el mecanismo por el que los AGPI, especialmente el linoleico, reprimen la expresión del gen *SCD*, mediante la unión a una región reguladora supresora en su promotor (Zulkifli *et al*., 2010). Los estudios previos coinciden también en las diferencias observadas entre tejidos, con una menor responsividad a la dieta del tejido muscular (Duran-Montgé *et al*., 2009b).

Estudios previos sugieren también un efecto represor del ácido oleico sobre la expresión del gen *SCD*, que es contrario a lo observado en nuestro trabajo, pues los animales alimentados con dieta rica en AGS acumularon más oleico en sus tejidos y presentaron a su vez mayor expresión para este gen. Esto podría sugerir un efecto bioactivo diferencial de los AG circulantes provenientes directamente del alimento, respecto de los sintetizados y acumulados en los tejidos. Por otra parte es un hecho conocido que el cerdo ibérico se caracteriza por una elevada capacidad de síntesis de AGMI, aparte de su también conocido mayor potencial lipogénico (López-Bote, 1998; Serra *et al*., 1998; Barea *et al*., 2013; Isabel *et al*., 2014; Óvilo *et al*., 2014b). También se ha descrito una mayor expresión del gen *SCD* en el músculo de animales ibéricos puros respecto a otros genotipos (Óvilo *et al.*, 2014b; Estany *et al*., 2014; Ayuso *et al.*, 2015b; Ayuso *et al*., 2016). Esta

mayor expresión podría ir acompañada de una regulación diferenciada en la que los efectos represores del ácido oleico fueran menos intensos, permitiendo así la mayor capacidad de desaturación y de acumulación de ácido oleico en los tejidos de los cerdos ibéricos.

El hígado y especialmente el tejido adiposo se han propuesto como los principales tejidos de síntesis endógena en el cerdo (Duran-Montgé *et al*., 2009b), y por lo tanto, se podría esperar una mayor influencia de la dieta sobre los genes lipogénicos en estos tejidos. Sin embargo solo observamos una mayor respuesta transcripcional en el hígado pero no en tejido adiposo, donde sólo se observó una respuesta sugestiva para el gen *SCD*. La mayor respuesta transcripcional observada en el hígado está en consonancia con un menor efecto de la dieta sobre la composición tisular de AG, sugiriendo una respuesta metabólica adaptativa en este tejido encaminada a mantener una composición en AG estable, como se deduce al no haber encontrado modificación alguna en la composición de AG de la fracción de lípidos neutros en el hígado.

Los genes *CPT1* y *HADH* están involucrados en el transporte y la degradación mitocondrial de los AG. Cabría esperar que un aumento de AGPI en la dieta se relacionara con una mayor función de las vías relacionadas con el transporte y la β-oxidación de AG de cadena larga en las mitocondrias, especialmente en tejidos ricos en estas organelas, como el cardiaco (Cordero *et al*., 2011; Vicente *et al*., 2013). Sin embargo, no detectamos ninguna diferencia en la expresión génica condicionada por la dieta para estos dos genes en ninguno de estos músculos, aunque la regulación de estas mismas enzimas a otros niveles no debería descartarse.

En este trabajo, así como en Óvilo *et al.*, 2014, la respuesta transcripcional se estudió en un solo estadío final y aplicando un tratamiento nutricional a largo plazo. Los efectos observados fueron modestos en número y magnitud y podrían estar indicando una adaptación metabólica progresiva a las dietas recibidas, por lo que se consideró necesario para estudios posteriores el empleo de tratamientos a corto o medio plazo. Teniendo este aspecto en consideración, y con el objetivo de profundizar en los efectos de la fuente de energía de la dieta sobre el metabolismo lipídico del tejido adiposo del cerdo ibérico, llevamos a cabo el segundo estudio de esta tesis. Teniendo en cuenta los resultados de los trabajos previos, se realizó un abordaje de gen candidato más amplio, con un mayor

número de genes analizados, incluyendo el estudio secuencial de la expresión génica a dos edades, así como evaluando la influencia de la situación fisiológica del animal en relación al consumo de alimentos (comparación de estados ayuno *vs* postprandial).

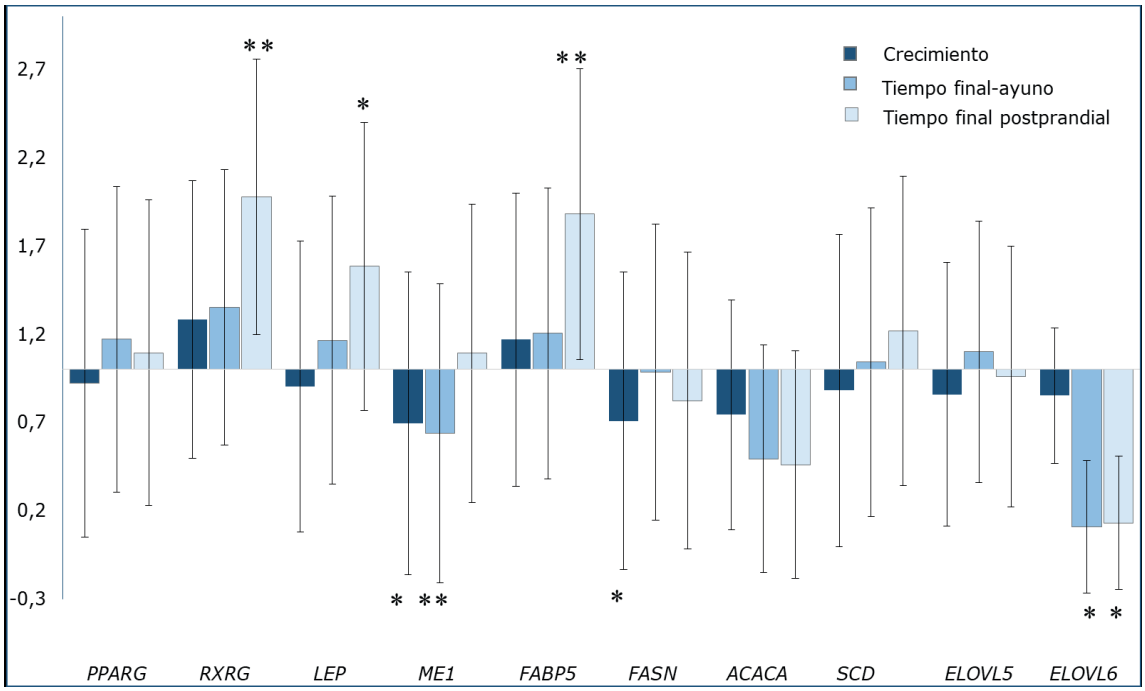
Al estudiar el efecto del período de crecimiento sobre la expresión génica pudimos comprobar que su efecto era importante en los genes relacionados con la lipogenésis. Ocho de los 10 genes candidato analizados se vieron afectados significativamente por el período de crecimiento (44 kg de peso vivo *versus* 100 kg de peso vivo), con la mayoría de ellos mostrando una mayor expresión a tiempo final. Además, la magnitud de las diferencias de expresión fue considerable en algunos genes como es el caso del gen *LEP*. Estos resultados coinciden con otros descritos por Hood y Allen (1973), en una línea porcina obesa, con una mayor actividad de enzimas lipogénicas en animales de más días de edad y más pesados. El gen *PPARG* fue el único que mostró mayor expresión en los animales jóvenes, en consonancia con su papel regulador en la diferenciación de los adipocitos, que ocurre en estadios tempranos de crecimiento (Tontonoz *et al.,* 1994).

En cuanto a la respuesta transcripcional a la intervención nutricional, ésta estuvo modulada sólo ligeramente a lo largo del tiempo y mostró un aumento en la expresión de varios genes lipogénicos en el grupo CH (*ME1, ELOVL6, ACACA*), confirmando una mayor síntesis *de novo* de AG en este grupo. Los resultados no fueron del todo concordantes con los obtenidos previamente (Óvilo *et al.*, 2014a), posiblemente debido al tipo de muestras analizadas: biopsias de tejido adiposo subcutáneo de jamón en este trabajo (incluidas ambas capas) y capa interna de grasa dorsal en el trabajo previo; dificultando así la comparación. Estos resultados resaltan la dificultad que supone la elección de la ubicación anatómica del tejido en este tipo de ensayos nutrigenómicos y en general en los estudios de expresión génica.

El ayuno, incluida la privación de alimentos a corto plazo, reduce la lipogénesis e induce la lipolisis (Kersten, 2001; Palou *et al*., 2010). Sorprendentemente, en nuestro trabajo, el ayuno solo afecto de forma sutil a los niveles de expresión. Solo el gen *PPARG* se vio afectado significativamente por un ayuno de 18h, aumentando su expresión 3h después de la ingestión de alimentos (postprandial). En trabajos previos se ha mostrado como la expresión del gen *PPARG* disminuye con el ayuno, estando este cambio acompañado de la represión de otros regula-

dores y enzimas lipogénicas (Houseknecht *et al*., 1998; Morgan *et al*., 2008). La falta de efectos del ayuno sobre la expresión de las enzimas lipogénicas incluidas en nuestro trabajo fue un resultado inesperado que podría estar relacionado con la duración del ayuno aplicado. Probablemente, después de periodos cortos o medios de ayuno, los reguladores de la lipogénesis podrían verse afectados, mientras que los períodos de ayuno más largos podrían conducir a una mayor respuesta en los genes regulados por dichos reguladores. Por ejemplo, un ayuno prolongado (72h) produce un mayor efecto sobre la expresión del gen *LEP* que sobre el gen *PPARG* en el cerdo (O'Gorman *et al*., 2010). Nuestros resultados también podrían ser consistentes con los que indican que, en ratones obesos, la lipogénesis *de novo* persiste en el hígado y tejido adiposo durante el ayuno (Morgan *et al*., 2008). Esta persistencia de la lipogénesis durante el ayuno, con independencia de la dieta, podría ser característica de la raza ibérica. La comprobación de esta hipótesis requería contrastar los efectos de ayuno en cerdo ibérico frente a otras razas magras, lo cual fue estudiado en el tercer trabajo de esta tesis.

Figura 15. Valores de expresión (FC) de los genes candidato entre dietas HO *vs.* CH en biopsias de tejido subcutáneo del jamón obtenidas en crecimiento y a tiempo final, en situaciones de ayuno y postprandial. Las líneas de error indican el error estándar estimado para los valores de FC. Valores de FC >1 indican mayor expresión en la dieta HO **p* <0.05, ***p* <0.01. HO = dieta enriquecida en girasol alto oleico; CH = dieta con carbohidratos como fuente de energía y sin grasa añadida.



De acuerdo con nuestros resultados de este segundo trabajo, y en concordancia con lo expuesto previamente, el gen *SCD* no mostro cambios de expresión en respuesta a la fuente de energía ni tampoco en respuesta al ayuno. Este resultado indicaría una expresión estable del gen *SCD* e independiente de distintos factores nutricionales, pues su expresión solo se vería inhibida por los AGPI de la dieta, pero no por el ácido oleico (tanto suministrado con el alimento como sintetizado en los tejidos) o el ayuno. Estos resultados observados en cerdos ibéricos contrastan con resultados observados en otras especies y otras razas porcinas (Duran-Montgé *et al*., 2009; Paton y Ntambi, 2009; Zulkifli *et al*., 2010) y refuerzan la hipótesis planteada previamente relativa a la posible regulación diferenciada del gen *SCD* en cerdo ibérico, con un menor efecto de factores represores de la expresión de dicho gen, que facilitaría la característica acumulación de ácido oleico en sus tejidos. Esta regulación diferenciada podría estar relacionada con cambios estructurales en las regiones reguladoras del gen, como por ejemplo el polimorfismo SCD.g.2228T (Estany *et al*., 2014), localizado en el promotor y cuyo alelo T, con alta frecuencia en la raza ibérica (Muñoz *et al*., 2018), está asociado a una mayor capacidad de desaturación de grasa. En cualquier caso, no debería descartarse la regulación de la expresión de la proteína SCD a otros niveles de actividad (Doran *et al*., 2006).

En referencia al objetivo 1 de esta tesis y considerando los resultados de los dos primeros trabajos, se observa que tanto a corto/medio plazo como a tiempo final, existen adaptaciones transcripcionales en los tejidos adiposo subcutáneo del jamón y hepático en respuesta a intervenciones nutricionales con distinta fuente de energía. Los resultados son coherentes en todos los tiempos analizados, a pesar de las fuertes diferencias en la expresión de los genes candidato entre periodos de crecimiento, y son compatibles con una mayor síntesis endógena *de novo* de AG en el grupo suplementado con carbohidratos y mayor desaturación en animales suplementados con grasa saturada. Los resultados en relación al estado de alimentación (ayuno y postprandial) sugieren una persistencia de la lipogénesis *de novo* durante el ayuno, lo que podría ser un rasgo característico asociado al fenotipo graso de los animales de raza ibérica. La exploración de los efectos transcripcionales de estas intervenciones con diferentes fuente de energía, los efectos del período de crecimiento y la situación fisiológica del animal han contribuido a profundizar en la comprensión de la regulación de la lipogénesis en

la raza ibérica. Los hallazgos tienen interés científico por el avance en el conocimiento de estos procesos que son de gran relevancia para la producción porcina y también en salud humana. Por otra parte, los datos de los efectos de la dieta sobre la composición tisular tienen interés aplicado por el potencial empleo de distintas fuentes de grasa para la formulación de la dietas.

4.3. EFECTOS DE LA DIETA Y LA RAZA SOBRE LA COMPOSICIÓN TISULAR Y LA EXPRESIÓN GÉNICA E INFLUENCIA DE LAS INTERACCIONES GENÉTICA-NUTRICIÓN

Los trabajos realizados en el marco del primer objetivo de esta tesis aportan información sobre aspectos metabólicos importantes del cerdo ibérico, con repercusión en caracteres productivos, y relacionados con el manejo y alimentación de los animales. Como complemento, el segundo objetivo de esta tesis pretendía profundizar en aspectos que no habían podido ser clarificados con los diseños empleados previamente. Estos aspectos incluyen los mecanismos específicos subyacentes al alto potencial lipogénico y de desaturación de los cerdos ibéricos y su potencial respuesta diferencial a la dieta en relación a otras razas.

Para este objetivo se diseñó un experimento (experimento 3, figura 13) generando un nuevo material animal que se exploró mediante las estrategias complementarias del gen candidato, incluyendo genes lipolíticos, lipogénicos y relacionados con la adipogénesis (capítulo 3), y del análisis del transcriptoma mediante secuenciación masiva de ARN (capítulo 4). En este experimento se utilizó una intervención nutricional similar a la utilizada en el segundo trabajo (HO *vs* CH) (dietas isoproteicas e isoenergéticas), se aplicó un periodo de ayuno prolongado (24 horas) y se amplió el estudio a dos razas (ibérica *vs* duroc) en pureza y en idénticas condiciones de manejo, en animales en crecimiento (hasta 50kg de peso vivo).

La literatura científica previa se ha centrado principalmente en la comparación de los dos tipos genéticos de interés productivo en el ámbito del cerdo ibérico (ibéricos puros y cruzados con duroc). Estos dos tipos genéticos muestran importantes diferencias en crecimiento, composición tisular y desarrollo muscular y adiposo, aportando la raza duroc una mejora en cuanto al rendimiento productivo pero no siendo así en cuanto a la calidad de su carne. Las diferencias entre estas razas están determinadas fundamentalmente por factores genéticos. Sin

embargo, el conocimiento sobre los mecanismos moleculares implicados en las diferencias en la calidad de su carne es todavía muy limitado, especialmente en la comparación de razas puras.

Para estos trabajos se escogió como tejido diana la grasa subcutánea del jamón, cuyo desarrollo y composición son esenciales en la producción del jamón curado, producto principal del cerdo ibérico.

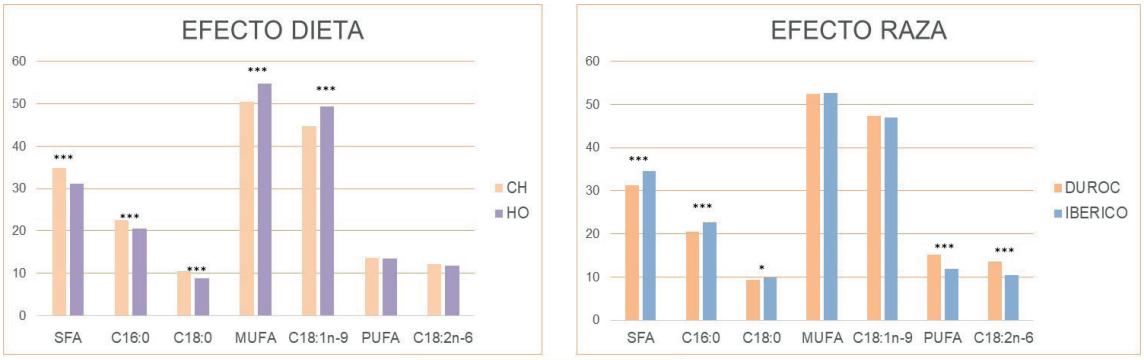
4.3.1. Efectos de la raza y la dieta a nivel de parámetros productivos y composición tisular

Ambas razas difirieron claramente en el engrasamiento y el apetito, en concordancia con estudios previos (Ventanas *et al*., 2006; Olivares *et al*., 2010; Fuentes *et al*., 2014). Sin embargo, es interesante remarcar que estos animales no presentaron diferencias significativas en peso corporal, aunque sí en el peso del jamón. Aunque muchos estudios han descrito diferencias en peso entre genotipos ibéricos puros y cruzados, al nacer y en la edad final de sacrificio, las diferencias en el peso corporal y el tamaño no son evidentes en el destete (Óvilo *et al*., 2014b) ni en los períodos de crecimiento temprano (Ayuso *et al*., 2016), coincidiendo con nuestros resultados. Este hecho podría asociarse con el genotipo ahorrador de los cerdos ibéricos, que podría conducir a altos niveles de ingesta voluntaria de alimento o a un bajo gasto de energía durante la lactación y períodos de crecimiento tempranos, produciendo un crecimiento compensador en esta fase (Ayuso *et al*., 2016).

Los efectos de la raza sobre la composición de AG de los tejidos fueron significativos. Se observó mayor contenido en AGS y menor de AGPI en la raza ibérica en comparación con la duroc. Por otra parte, el nivel de AGPI en los tejidos porcinos solo depende de su nivel en la dieta ingerida al tratarse principalmente de AG esenciales, por lo tanto, los niveles significativamente más altos de AGPI encontrados en el tejido adiposo de los cerdos duroc podrían explicarse por una mayor capacidad de estos animales para almacenar lípidos insaturados de la dieta en sus tejidos (Ventanas *et al*., 2006). Los resultados de composición indicarían que el empleo de una dieta suplementada con ácido oleico en la raza duroc podría ser una herramienta útil para mejorar la calidad sensorial, organoléptica y nutricional de sus productos cárnicos.

Los resultados del efecto de la dieta a nivel de composición fueron consistentes con los encontrados en los dos primeros trabajos. Las dos localizaciones estudiadas (grasa subcutánea dorsal y grasa subcutánea del jamón) respondieron de

Figura 16. Porcentaje de los principales AG de la grasa subcutánea del jamón.



forma similar y reflejaron al menos en parte la composición de la dieta recibida, con mayor contenido de AGMI en el grupo de alto oleico. El contenido de AGS fue mayor en el grupo alimentado con carbohidratos, a pesar de que esta dieta incluyó una menor proporción de ácidos palmítico y esteárico, indicando la síntesis *de novo* de AG a partir de los carbohidratos disponibles en la ración, al igual que se observó en el ensayo previo. El contenido de AGPI fue consistente con la deposición directa de estos AG. Todos estos resultados coincidieron con trabajos anteriores de nuestro grupo y de otros autores (Ventanas *et al*., 2008; Óvilo *et al*., 2014). La suplementación con aceite de girasol alto oleico redujo la saturación de la grasa del cerdo y el ratio n-6/n-3, repercutiendo de forma positiva en la calidad nutricional de la carne en las dos razas.

4.3.2. Efectos de la raza, la dieta y el ayuno sobre la expresión génica

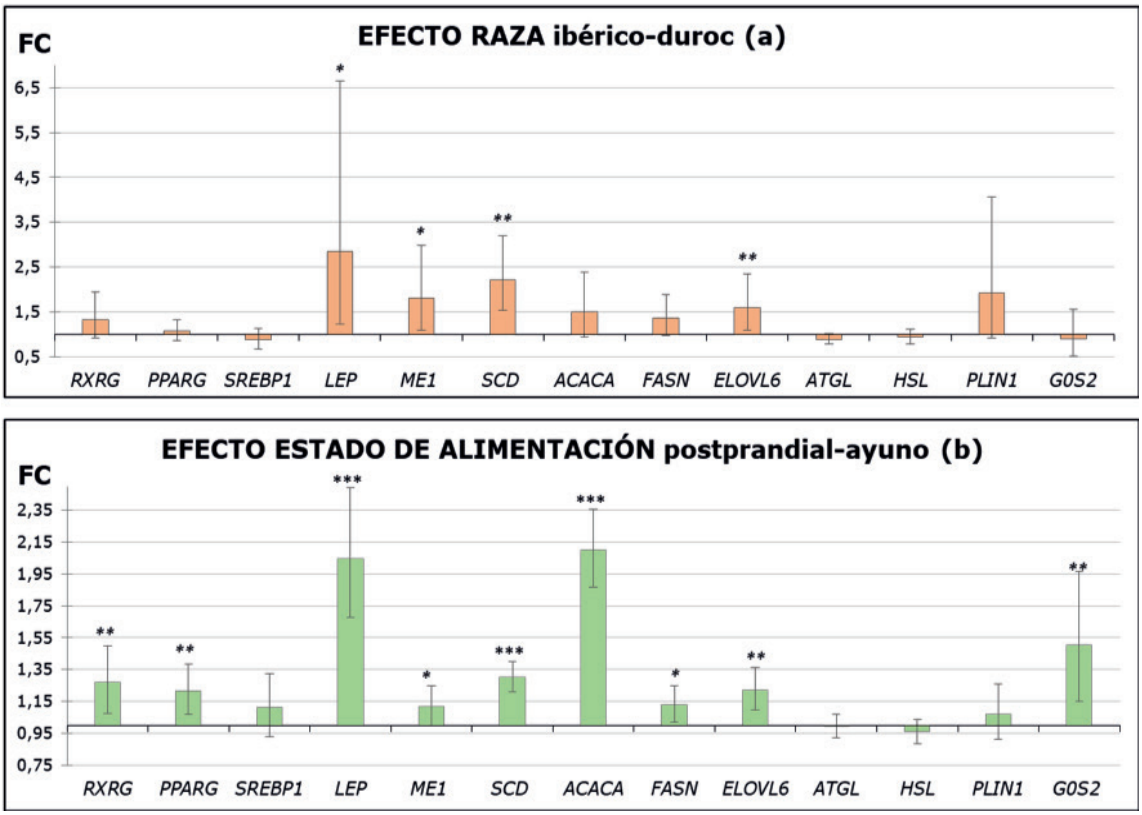
Los resultados de expresión génica del panel de genes candidatos analizados en el tercer trabajo de la tesis nos proporcionaron una primera idea de la regulación diferencial existente entre razas. De hecho, la raza fue el factor con mayor influencia sobre la expresión génica tanto en el abordaje de gen candidato como a nivel del transcriptoma del tejido adiposo del jamón.

En la aproximación de gen candidato la raza afectó a aquellos genes implicados en el balance energético y la lipogénesis, que estuvo lógicamente aumentada en la raza ibérica. El gen de la leptina presentó la mayor sobreexpresión en la raza ibérica, hallazgo que se detectó en ambos abordajes (FC=2.85 en gen candidato y FC= 2.13 en RNAseq) de acuerdo con sus altos niveles de proteína

detectados en ibérico (Fernández-Figares *et al*., 2007). El resto de genes sobrexpresados en la raza ibérica estuvieron directamente implicados en la lipogénesis *de novo*, en consonancia con nuestros resultados previos y con las características de la raza (López-Bote *et al*., 1998; Serra *et al*., 1998; Barea *et al*., 2013; Isabel *et al*., 2014). El gen *SCD* también estuvo sobreexpresado de forma significativa en la raza ibérica (FC=2.21) coincidiendo con los resultados de una comparación previa del transcriptoma muscular entre cerdos ibéricos y cruzados con duroc (Óvilo *et al*., 2014b). Este resultado también concuerda con la segregación del polimorfismo de tipo SNP, mencionado previamente y localizado en el promotor del gen *SCD*, AY487830: g.2228T> C (Estany *et al*., 2014). Este polimorfismo tiene un alelo (g.2228T) asociado con una mayor expresión del gen y que es mayoritario en el cerdo ibérico pero que segrega en la raza duroc. Esta regulación positiva del gen *SCD* en los cerdos ibéricos puede estar asociada con el conocido mayor potencial de desaturación de esta raza (Fernández *et al*., 2017) aunque, en nuestro trabajo, esta diferencia en la expresión génica no se tradujo en un mayor contenido de AGMI. Una posible hipótesis para explicar este hecho es la mayor ingesta en cerdos ibéricos y el predominio de la lipogenesis *de novo* sobre la desaturación de los AG en el período de crecimiento, lo que podría conducir a un intenso incremento en AGS y a una dilución de los demás AG.

No detectamos ningún efecto de la raza sobre la transcripción de los reguladores involucrados en la diferenciación de adipocitos (RXRG, PPARG, SREBP1), en contraste con los hallazgos previos en animales más jóvenes (Garcia-Contreras *et al.*, 2017), probablemente debido al momento del muestreo con respecto a los procesos de diferenciación celular. Conjuntamente, los efectos encontrados para la raza sobre los genes lipogénicos concuerdan con la predisposición a la obesidad de la raza ibérica, proporcionando una base molecular para el desarrollo de resistencia a la leptina y síndrome metabólico en los cerdos ibéricos alimentados con dietas altas en grasas (Torres-Rovira *et al*., 2012), y refuerzan la utilidad de este cerdo como modelo biomédico para estudiar la obesidad y otros trastornos metabólicos. Además, nuestros hallazgos en este trabajo en relación a la activación de la lipogenesis *de novo* en la raza ibérica han sido confirmados en el último trabajo de esta tesis, mediante la comparación del transcriptoma de estas dos razas, en la que hemos observado una activación de genes, rutas y reguladores implicados en el metabolismo lipídico.

Figura 17: Ratios de expresión (FC) de los genes candidatos en el tejido adiposo subcutáneo del jamón entre a) ibérico *vs* duroc b) postprandial *vs* ayuno. Las barras de error indican los errores estándar. Valores de FC>1 indican una expresión mayor en ibérico y en estado postprandial. *p<0.05, **p<0.001, ***p<0.0001.



En relación al efecto del ayuno, el panel de genes estudiado aumentó de los 10 utilizados en el segundo trabajo a 13 en éste, al incluirse genes relacionados con los procesos de lipolisis que podrían contribuir a una mejor comprensión de los efectos del ayuno (Figura 17). Nueve de los trece genes analizados se vieron afectados significativamente por el estado de alimentación, estando todos ellos sobrexpresados en el estado postprandial.

Específicamente, el consumo de alimentos activó todos los genes lipogénicos (*SCD, ME1, ACACA, FASN, ELOVL6* y *LEP*), dos reguladores de la adipogénesis (*RXRG* y *PPARG*), y un gen con un papel de regulador negativo de la lipolisis (*GOS2*). Aunque este último gen está implicado funcionalmente en la lipolisis, es un regulador inhibidor de esta, y hay estudios previos que también indican una regulación negativa de este gen después de 24 h de ayuno (Zhang *et al*., 2014).

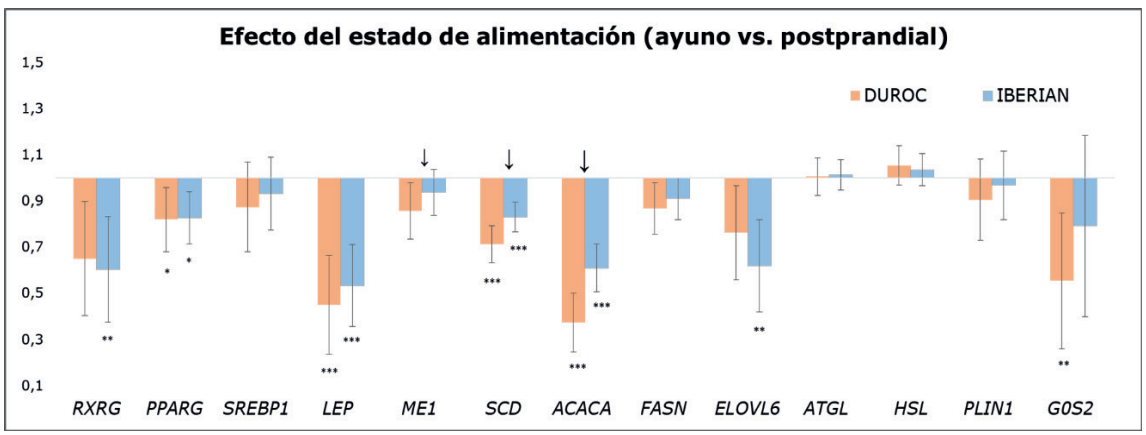
La acumulación grasa resulta del balance entre lipogénesis y lipolisis, por lo que el estudio de enzimas lipolíticas en respuesta a la dieta o al ayuno podría ayudar a esclarecer los mecanismos de regulación del metabolismo en tejidos

ibéricos. Nuestros resultados coinciden claramente con los efectos esperados del ayuno de inhibición de la lipogénesis y de la diferenciación de adipocitos. Respecto a los procesos de lipolisis se observó, que después de un ayuno de 24 h, más largo que el utilizado en el segundo trabajo (18h), los principales genes lipolíticos *ATGL, HSL*, *PLIN1* no se vieron afectados.

La comparación de resultados obtenidos con ayunos de 18 y 24 horas muestra un efecto mucho mayor en el segundo, confirmando que la falta de efectos observada tras 18h de ayuno podía estar relacionada, al menos en parte, con la duración del ayuno aplicado, de acuerdo con lo indicado en otros trabajos (Morgan *et al*., 2008; O'Gorman *et al*., 2010). Se puede deducir por tanto que en los cerdos ibéricos es preciso un periodo de ayuno prolongado (24h) para inhibir la lipogénesis.

Por otro lado, nuestros resultados anteriores sugirieron que, en los cerdos ibéricos, como también ocurre en ratones obesos, la lipogénesis *de novo* puede ser más persistente en el tejido adiposo durante el ayuno que en animales magros (Morgan *et al*., 2008). Para probar esta hipótesis, los efectos de la raza y del ayuno se consideraron conjuntamente, observándose efectos significativos de interacción entre los efectos de la raza y el ayuno para los genes *SCD* y *ACACA*, y obteniéndose un resultado similar de significación sugestiva para el gen *ME1*. En los tres casos, las interacciones fueron cuantitativas y revelaron que la inhibición de estos genes después de 24 h de ayuno fue más intensa en los cerdos duroc, o en otras palabras, su expresión fue más estable en los cerdos ibéricos (Figura 18).

Figura 18. Ratios de expresión (FC) entre los estados fisiológicos ayuno *vs* postprandial de los genes candidato en las biopsias de tejido adiposo obtenidas en cerdos ibéricos y duroc. Las líneas de error indican los errores estándar. FC>1 indican mayor expresión en ayuno.*p<0.05, **p<0.01, ***p<0.0001. Las interacciones raza*estado están indicadas con flechas (↓) (p<0.07, p<0.03 y p<.0001 para *ME1, SCD* y *ACACA* respectivamente).



Además, los patrones de expresión de otros genes lipogénicos (*SREBP1, LEP, FASN*) fueron similares, con un efecto del ayuno más pronunciado en los cerdos duroc. Aunque los efectos de interacción para algunos genes no alcanzaron la significación estadística, conjuntamente, todos estos resultados apoyan una lipogénesis más persistente en el ibérico que en la raza duroc. Esta es una información novedosa sobre la regulación de la deposición de grasa en relación con la obesidad determinada genéticamente, que hasta ahora se basaba en los escasos resultados encontrados en ratones con una obesidad inducida por la dieta (Morgan *et al*., 2008). Además, nuestros resultados extienden el estudio a un periodo de ayuno (24 h) *versus* un ayuno nocturno (18 h), y a nuevos genes como *SCD, ME1,* y *LEP*.

En cuanto a la suplementación de la dieta con ácido oleico o carbohidratos, sorprendentemente, no se observó efecto sobre la expresión de ninguno de los genes analizados, excepto el gen *PLIN1*, que estuvo sobreexpresado en el grupo CH. Estos escasos efectos fueron inesperados, ya que se habían observado efectos con dietas similares, en los cerdos ibéricos en etapas iniciales y finales de crecimiento, en el segundo trabajo de esta tesis y en Óvilo *et al*., 2014. Sin embargo, no hay que olvidar que los efectos de las intervenciones nutricionales con AG sobre la expresión génica son escasos, pequeños y difíciles de medir, y además estos efectos están profundamente modulados por diferentes factores como el genotipo, el tejido o el momento de muestreo. Por tanto, los efectos de interacción podrían haber condicionado nuestra capacidad para detectar los efectos de la dieta. Ya en el segundo trabajo de esta tesis, encontramos evidencia de una interacción significativa de la dieta*ayuno sobre la expresión del gen *ME1*. En este trabajo, las interacciones cualitativas de la dieta*ayuno se extienden a los genes *ACACA, SREBP1, ATGL, HSL* y *PLIN1*. En cuanto a los genes *ACACA* y *SREBP1*, los efectos de la dieta sobre su expresión parecían ser opuestos entre los estados de ayuno y postprandial. De igual manera, los genes lipolíticos *ATGL, HSL y PLIN1*, mostraron una interacción cualitativa similar, con el mismo patrón para los tres genes, pero con una tendencia opuesta a la de los genes *ACACA* y *SREBP1*. Estas interacciones pueden estar relacionadas con diferencias en la dinámica de absorción y la disponibilidad de los nutrientes en sangre en estado postprandial entre las dietas utilizadas, así se esperaría que la dieta CH aumente los niveles de glucosa de forma más rápida que la dieta HO.

Aunque todos estos resultados deben considerarse con cautela debido a la falta de significación estadística para algunos genes, la coincidencia en los patrones observados para los genes relacionados funcionalmente sugiere una base biológica subyacente que debe ser explorada en trabajos futuros. Por ejemplo, los tres principales genes lipolíticos, *ATGL, HSL y PLIN1*, presentan exactamente el mismo patrón de interacción dieta*raza y dieta*ayuno, de acuerdo con su papel biológico común.

Las interacciones encontradas justifican plantear una hipótesis de respuesta diferencial entre razas a los factores nutricionales. En consecuencia, y utilizando el mismo material experimental que en el tercer trabajo, utilizamos un enfoque de análisis del transcriptoma con el objetivo de realizar un análisis más completo de los efectos de la raza, la fuente de energía de la dieta y su posible interacción sobre la expresión génica y el metabolismo del tejido adiposo.

El efecto de la raza sobre el transcriptoma del tejido adiposo fue mayor al de la dieta, en número de genes DE así como en la magnitud de las diferencias de expresión, en concordancia con los hallazgos previos obtenidos en el estudio de genes candidato. Los datos transcriptómicos confirmaron un intenso efecto de la raza sobre el transcriptoma, afectando a la expresión de genes candidato relacionados con el metabolismo lipídico y la homeostasis energética previamente analizados y que tenían una regulación positiva en la raza ibérica como por ejemplo los genes *LEP, ME1* o *RXRG*, y nos proporcionaron información de otros muchos genes DE, relacionados con estos procesos. Es necesario considerar que algunas de las diferencias encontradas entre razas podrían estar reforzadas por las diferencias de apetito entre ambas razas, lo que por otra parte podría ser característico de cada una de ellas.

En la raza ibérica el análisis funcional de los genes DE mostró una activación de procesos y reguladores relacionados principalmente con la respuesta inflamatoria y el metabolismo lipídico y de los carbohidratos. La activación de genes y el enriquecimiento de funciones relacionadas con el metabolismo de la glucosa y el metabolismo lipídico en la raza ibérica es un resultado esperable que puede indicar un mayor uso de los glúcidos de la dieta para la síntesis *de novo* de lípidos y una mayor acumulación de grasa de acuerdo con el fenotipo característico de los animales de esta raza (Nieto *et al*., 2002; Fernández-Figares *et al*., 2007; Switonski *et al*., 2010), confirmando todos nuestros resultados previos, ahora a nivel del transcriptoma de este tejido. Sin embargo, los resultados encontrados en relación con la respuesta inflamatoria fueron novedosos. La acumulación grasa que

se da en el TA de animales obesos se relaciona con alteraciones metabólicas que incluyen un estado de inflamación crónica de bajo grado (Gustafson *et al*., 2007 y Pahlvani *et al*., 2017). En el cerdo ibérico, nuestros resultados de interpretación funcional encajan en gran medida con una activación de los procesos relacionados con la infiltración y acumulación de varios tipos de células inmunes asociados a la inflamación, y compatibles con una inflamación de bajo grado desarrollada como consecuencia de la acumulación de lípidos. Las alteraciones metabólicas de la obesidad se asocian también con una pérdida de sensibilidad a la insulina tanto de forma local en el TA como a nivel sistémico (Gutiérrez *et al*., 2009 y Lee and Lee, 2014). Los datos transcriptómicos apoyan también el desarrollo temprano de resistencia a insulina en los animales de raza ibérica. Aunque el cerdo ibérico se considera un adecuado modelo animal para estudios de engrasamiento, obesidad y enfermedad metabólica, la existencia de procesos inflamatorios del tejido adiposo asociados al engrasamiento no se ha descrito anteriormente en esta raza. Por el contrario, un estudio reciente muestra una profunda inflamación así como necrosis del TA visceral, aunque no del TA subcutáneo en cerdos obesos *Göttingen minipigs* (Renner *et al.*, 2018), que se hacen evidentes después de un largo periodo de alimentación con dieta rica en grasa. El desarrollo precoz de este tipo de procesos inflamatorios en el cerdo ibérico puede resultar inesperado, aunque se ha visto en la especie humana que las alteraciones tisulares asociadas a obesidad pueden empezar a aparecer desde estadios tempranos de su desarrollo (Singer & Lumen, 2017). Nuestros animales, ya desde la etapa de crecimiento, mostraron grandes diferencias en el desarrollo de este tejido que resultaron en valores extremos del espesor de tocino (24.1 mm *vs*. 10.7 mm para la grasa dorsal y 27.8 mm *vs*. 15.7 mm para la grasa del jamón en ibérico y duroc respectivamente, $p < 0.001$). Estos datos concuerdan con la predisposición genética al engrasamiento y desarrollo de la inflamación y señalarían de nuevo a este animal como un modelo biomédico para la obesidad y sus trastornos metabólicos asociados. En este sentido, los estudios secuenciales del tejido adiposo a nivel histológico pueden ayudar a comprender en el futuro la progresión y magnitud de estos fenómenos, como complemento al estudio de su base molecular.

En contraste, en la raza duroc los resultados de la interpretación funcional de los genes DE mostraron una activación de procesos relacionados con el crecimiento, desarrollo y organización celular y en general con todos aquellos proce-

sos relacionados con el crecimiento del animal. A nivel del transcriptoma muscular, estas diferencias en la activación de los procesos de desarrollo y crecimiento celular ya habían sido detectadas en comparaciones entre animales puros y cruzados (Óvilo *et al*., 2014b y Ayuso *et al*., 2016). Sin embargo, a nivel del tejido adiposo se esperaría que la raza ibérica tuviese un mayor potencial, al menos en relación a la diferenciación de células adiposas. Nuestros resultados indicarían por tanto una respuesta coordinada en la regulación general de los procesos del desarrollo en los distintos tejidos acorde al mayor potencial de crecimiento de la raza duroc. En esta raza también se observó un enriquecimiento de funciones y redes relacionados con los procesos de organización del esqueleto y la matriz extracelular indicando en esta raza una activación de procesos preponderantemente constructivos y relacionados con una matriz extracelular más densa coincidiendo con resultados similares encontrados en otros estudios (Horodyska *et al*., 2019).

Debido al enorme efecto de la raza sobre el transcriptoma del TA se estudió el efecto de la dieta por separado en cada raza. La raza ibérica mostro un mayor efecto de la dieta en comparación con la raza duroc tanto en número de genes DE como en la magnitud de las diferencias de expresión.

En la raza ibérica, el análisis funcional del efecto de la dieta sobre el transcriptoma mostró también un enriquecimiento de procesos inflamatorios, del metabolismo lipídico, del metabolismo y señalización de corticoides y del desarrollo del tejido graso, en consonancia con todos los hallazgos encontrados hasta ahora en esta raza. En relación al metabolismo lipídico y de carbohidratos, todos nuestros resultados, tanto de este último trabajo como de los anteriores, estarían de acuerdo con la presencia de niveles más altos de carbohidratos en el grupo de la dieta CH y su empleo para la síntesis endógena de lípidos. Por otra parte los genes activados en la dieta HO se relacionan con el metabolismo de los lípidos y el colesterol, la glucólisis y la gluconeogénesis, lo que podría indicar el empleo de los AG de la dieta como fuente de energía.

Los resultados indican el desarrollo de una inflamación de bajo grado en el TA de los dos grupos experimentales de la raza ibérica aunque se observan diferentes procesos y tipos celulares implicados en cada uno. Atendiendo a los términos funcionales y los tipos de células específicamente activados en los dos grupos podría interpretarse que la dieta HO indujera una respuesta inflamatoria más intensa o más temprana en comparación con la dieta CH. Este potencialmente distinto ritmo

de los procesos de inflamación entre ambas dietas parece indicar el desarrollo desacompasado del proceso inflamatorio, lo cual podría estar relacionado con el efecto diferencial de los nutrientes recibidos en la dieta y presentes en la circulación (grasa en la dieta HO *vs* glucosa en la dieta CH), así como de los distintos tipos de AG que se depositan en el TA de los animales de ambas dietas (AGMI en HO *vs* AGS en la dieta CH), que influyen de forma diferencial en el desarrollo de la inflamación inducida por la obesidad (Teng *et al*., 2014). Este efecto bioactivo diferencial sobre la expresión génica, con un efecto más intenso de los AG circulantes respecto a los depositados, seria concordante con nuestros resultados del primer trabajo relativos a la falta de efecto represor de los AGMI sintetizados en los tejidos de animales alimentados con AGS sobre la expresión del gen *SCD*.

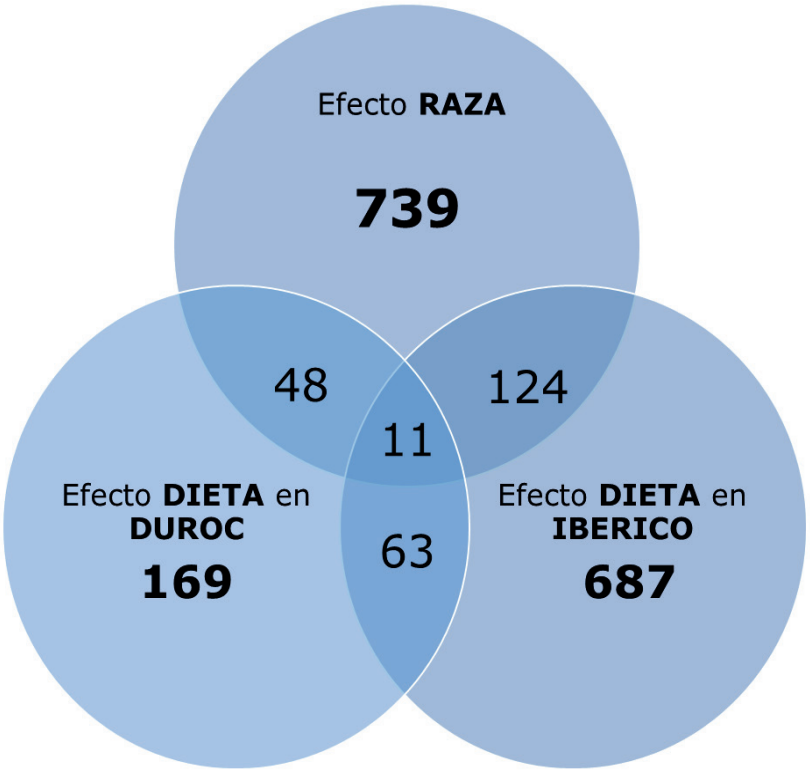
En los cerdos de la raza duroc, el efecto de la dieta sobre el transcriptoma fue muy pequeño en comparación con la raza ibérica. La interpretación funcional de los genes DE fue mucho menos clara que en ibérico. Aunque algunas categorías funcionales coincidieron, no se observó una activación o inhibición clara en la mayoría de las funciones y redes génicas. Es interesante remarcar que algunas funciones biológicas relacionadas con el tráfico de células inmunitarias, como son el reclutamiento de leucocitos o su migración, mostraron una respuesta opuesta a la dieta en ambas razas, activándose en HO en ibérico, pero en CH en duroc.

El volumen de datos generados en estudios transcriptómicos es enorme y en este estudio se han identificado un elevado número de genes DE que podrían tener interés como genes candidato susceptibles de estudios futuros más específicos, encaminados a desentrañar su papel en el fenotipo. Además de los genes DE, el análisis funcional de los datos transcriptómicos también nos permitió identificar potenciales reguladores responsables de las diferencias de expresión detectadas. Se identificaron un gran número de reguladores, especialmente para el efecto de la raza y para el efecto de la dieta en ibérico. En total, se identificaron 739 potenciales reguladores para el efecto de la raza, 687 para el efecto de la dieta en ibérico y 169 para el efecto de la dieta en duroc ($p<0.05$), por lo que la priorización de potenciales genes candidato es complicada. La comparación de los listados de reguladores correspondientes a los tres contrastes nos permitió identificar un número más reducido de reguladores comunes, incluyendo solamente 11 reguladores, que podrían estar implicados en el efecto de la raza y de la dieta en ambas razas (Figura 19). De los 11 reguladores comunes, la mayoría estu-

vieron relacionados con el crecimiento, la diferenciación y el crecimiento celular. Algunos de ellos estuvieron también implicados en la regulación de la adipogenesis. Podemos destacar entre ellos a MAPK1, relacionado con diferenciación adipocitaria y obesidad (Bost *et al*., 2005), y TP53, relacionado con la homeostasis y el metabolismo del TA (Krstic *et al*., 2018). Por otra parte es interesante señalar que en relación al efecto de la dieta observamos 63 reguladores comunes entre razas. Para estos 63 reguladores comunes la evidencia de regulación por la dieta es muy sólida al detectarse en paralelo en las dos razas. Por otra parte el elevado número de reguladores detectados en ibérico y no en duroc podrían explicar mecanismos de regulación específicos de la raza ibérica. La detección de estos reguladores abre nuevas vías de investigación en relación con los procesos de diferenciación adipocitaria y metabolismo y homeostasis del tejido adiposo.

En el análisis de los efectos raza y dieta encontramos varias evidencias de una potencial interacción entre estos efectos. Los genes afectados por la dieta en las dos razas fueron muy diferentes (207 genes DE afectados por la dieta en Ibérico

Figura 19. Diagrama de Venn que muestra los reguladores identificados como potenciales responsables de las diferencias de expresión observadas en los distintos contrastes (p<0.05).



REGULADORES COMUNES
CTNNB1
FH
HOXA10
NPPB
MAPK1
PROX1
TARBP2
TCF
TP53
TP73
UBE3A

y 58 en duroc y sólo siete genes DE comunes), y el rango de las diferencias de expresión fue mayor en los cerdos ibéricos que en los duroc. Todo ello indicaba una respuesta transcriptómica a la dieta diferente y más intensa en animales ibéricos que en los duroc. El empleo de la herramienta DESeq2, que permite analizar modelos con interacción, combinado con los resultados de la validación mediante qPCR, nos permitió inferir un número modesto de genes para los que la interacción podía considerarse estadísticamente significativa. Entre ellos, tiene especial interés el gen *SERPINE1* que además de estar DE en función de la dieta en ambas razas, mostró un efecto opuesto de la dieta en cada raza, sobreexpresado en la dieta HO en ibérico y en la dieta CH en duroc, indicando una clara interacción de tipo cualitativo. Este gen fue identificado como el regulador principal responsable del control transcripcional de una cascada de 30 reguladores y 62 genes DE, implicados principalmente en los procesos inflamatorios. También es interesante resaltar que la mitad de las interacciones que pudimos detectar en los distintos análisis fueron de tipo cualitativo, con una respuesta opuesta a la dieta en cada raza, al igual que la observada para el gen *SERPINE1*, cuya interpretación biológica es complicada. El resto de las interacciones detectadas fueron de tipo cuantitativo y se correspondían con genes que respondían a la dieta en la raza ibérica pero no en la duroc. La interpretación funcional de los genes afectados por la interacción dieta*raza mostró un enriquecimiento de las funciones y redes de señalización relacionadas con la respuesta inflamatoria, el movimiento celular y los trastornos de tipo metabólico.

Los efectos de interacción siempre son difíciles de medir e interpretar y nuestro análisis pone de manifiesto las dificultades para la identificación de estos efectos con las herramientas estadísticas disponibles para análisis de datos transcriptómicos. En cualquier caso nuestros resultados ponen de manifiesto la existencia de dichos efectos de interacción y nos permiten interpretarlos como una consecuencia del engorde diferencial de las dos razas que conduce a una clara respuesta inflamatoria en el tejido adiposo de la raza ibérica y cuya progresión podría estar a su vez influenciada por el tipo de dieta recibida.

4.4. REFLEXIONES FINALES Y PERSPECTIVAS DE FUTURO

Los trabajos comprendidos en esta Tesis Doctoral proporcionan una caracterización fenotípica y transcripcional de los procesos metabólicos de cerdos ibéricos y duroc criados en idénticas condiciones experimentales, especialmente centrada

en el tejido adiposo. Los resultados coinciden con el alto potencial lipogénico y de desaturación, el elevado apetito, el genotipo ahorrador y la resistencia a la leptina de la raza ibérica y respaldan la persistencia de la lipogénesis *de novo* durante el ayuno en este tipo de animales de fenotipo obeso. Los resultados en su conjunto profundizan en la base molecular del fenotipo característico de la raza ibérica y apoyan su utilidad como modelo animal para estudiar algunos tipos de obesidad y trastornos metabólicos, incluyendo el estudio de los procesos inflamatorios asociados a la acumulación de grasa en el tejido adiposo.

Nuestros resultados aportan también información relevante relativa a las consecuencias fenotípicas y transcripcionales de la suplementación de la dieta con carbohidratos y distintos tipos de AG. La caracterización fenotípica proporciona información novedosa e importante sobre la potencial utilidad de las intervenciones nutricionales empleadas para modular la composición tisular y la calidad del cerdo, no solo en la raza ibérica sino también en la duroc, en la que el margen de mejora en relación a la calidad de los productos es más amplio. A nivel transcripcional, la comparación de dietas aporta información interesante sobre los efectos bioactivos de los nutrientes empleados y la dificultad de su detección. Sin embargo, es necesario tener también en cuenta que los resultados obtenidos en los distintos experimentos están condicionados, no solo por las proporciones relativas de estos nutrientes en los que se ha forzado una diferencia importante entre dietas experimentales, sino también por otras ligeras variaciones en otros nutrientes con potencial bioactivo. Por ejemplo, el pienso enriquecido con girasol alto oleico presenta un contenido ligeramente superior en vitamina E, ácidos grasos ω-3 y fibra que el pienso de carbohidratos, lo que podría contribuir también a los efectos metabólicos de las dietas. Por otra parte, el contenido en ω-3 de todos los piensos empleados es bastante bajo (1.5%), como suele ocurrir en los piensos comerciales empleados en alimentación porcina, basados en cereales, lo que podría limitar la capacidad antioxidante y antiinflamatoria de los tejidos animales contribuyendo al desarrollo temprano del proceso de inflamación crónica en el cerdo ibérico. Es interesante resaltar que los procesos inflamatorios podrían estar, en consecuencia, exacerbados en las poblaciones ibéricas de manejo intensivo respecto a las de manejo tradicional y engorde en montanera, en las que el consumo de hierba supone un aporte muy superior de ácidos grasos ω-3. Estas consideraciones ponen de manifiesto las dificultades en la evaluación

aislada de los efectos de los nutrientes sobre el metabolismo y la necesidad de seguir profundizando en dichos efectos y evaluando nuevas y distintas estrategias, como por ejemplo la suplementación con ω-3, o el empleo combinado de distintos suplementos.

Por otra parte el abordaje transcriptómico empleado, basado en secuenciación masiva, ha generado un enorme volumen de datos de secuencia de regiones codificantes, que debe ser explorado en el futuro. El estudio de estos datos así como de posibles variantes estructurales en regiones reguladoras de los genes DE que pudieran asociarse con las diferencias de expresión observadas y con el fenotipo de nuestros animales es una importante línea de trabajo. Asimismo, la información relativa a la predicción de reguladores también debería ser empleada para la priorización de genes candidato ya que debido a su función reguladora estos podrían tener un profundo papel sobre el fenotipo. Esta vía de trabajo es complicada debido al enorme volumen de resultados disponibles. Una posible aproximación podría ser contrastar la posición de los reguladores detectados con la posición de QTLs y eQTLs detectados en porcino como regiones asociadas a rasgos o datos de expresión génica relacionados con características de desarrollo y composición del tejido adiposo, para priorizar los reguladores de mayor interés, que podrían ser objeto de estudios funcionales. De forma complementaria a la predicción de reguladores ya realizada, podría también plantearse el estudio de lncRNAs, para evaluar otras posibles vías de regulación de las diferencias de expresión y fenotípicas observadas.

Los ensayos desarrollados en esta tesis han proporcionado un material experimental amplio y de gran interés para estudios futuros. Por ejemplo, las muestras del músculo *biceps femoris* correspondientes al último diseño experimental se han procesado ya en paralelo a las de tejido adiposo, generando gran cantidad de información adicional fenotípica y transcripcional, aun no publicada, que no ha podido recogerse en esta tesis doctoral. El análisis completo de estos datos disponibles junto con el empleo de muestras de otros tejidos, como el hígado o las papilas gustativas, puede permitir explorar hipótesis complementarias para profundizar en las peculiaridades metabólicas del cerdo ibérico, como por ejemplo el posible mayor recambio proteico, que podría ser un condicionante principal del desarrollo muscular en esta raza, o la exploración de otros mecanismos moleculares relacionados con su mayor apetito.

5. CONCLUSIONES/ CONCLUSIONS

CONCLUSIONES

1. Los AG de la dieta, especialmente los AGMI y AGPI, se deponen en los tejidos con escasas modificaciones reflejándose en ellos en gran medida la composición de la dieta ingerida.

2. En la raza ibérica, tanto a corto/medio plazo como a tiempo final, las intervenciones nutricionales con carbohidratos y AG inducen adaptaciones transcripcionales en los tejidos adiposo y hepático.

3. Los glúcidos de la dieta estimulan la síntesis endógena de grasa saturada y los AGS estimulan la síntesis de AGMI, sugiriendo por tanto un papel activador de las dietas de carbohidratos y AGS sobre la lipogénesis *de novo* y la desaturación de AG.

4. El efecto del período de crecimiento sobre la expresión de los genes relacionados con el metabolismo lipídico es grande. Para los genes codificantes de enzimas lipogénicas la expresión aumenta con la edad. En cambio, el gen *PPARG* muestra una mayor expresión en los animales jóvenes en consonancia con su papel de regulador de la adipogénesis, que ocurre en estadios tempranos de desarrollo.

5. En el tejido adiposo del cerdo ibérico, tras un periodo de ayuno de 18h, solo se observa una adaptación transcripcional en el regulador PPARG, siendo preciso un periodo de ayuno más prolongado (24h) para para inducir una adaptación en la función de los principales genes lipogénicos.

6. Durante el ayuno, la lipogénesis *de novo* en el tejido adiposo es más persistente en los cerdos ibéricos que en los duroc, lo que puede considerarse un rasgo característico asociado al fenotipo graso de estos animales.

7. La regulación de los genes lipolíticos en el tejido adiposo, por factores como la raza, el estado de alimentación y la dieta, es muy compleja y está sujeta a intrincadas interacciones.

8. El efecto de la suplementación de la dieta con AG sobre el metabolismo lipídico es moderado y variable en función de otros factores como puede ser la raza o el estado de ayuno, resaltando la complejidad de su regulación y la dificultad en su estudio.

9. La raza tiene un efecto profundo sobre el transcriptoma del tejido adiposo. La mayoría de las diferencias transcripcionales encontradas entre las razas ibérica y duroc se relacionan con el crecimiento, la formación de la matriz extracelular, el metabolismo de los lípidos y de los carbohidratos y de forma remarcada con la respuesta inflamatoria e inmune. Algunas de estas diferencias pueden estar relacionadas con el mayor apetito de los animales ibéricos.

10. El tejido adiposo del cerdo ibérico en crecimiento se caracteriza por un perfil transcripcional compatible con el desarrollo de una inflamación de bajo grado y un síndrome de resistencia a la insulina, en concordancia con su elevada deposición grasa desde estadios tempranos de desarrollo. Estos resultados confirman el interés del cerdo ibérico como modelo biomédico para estudios de obesidad, trastornos metabólicos e inflamación de tejido adiposo.

11. Los efectos de la dietas suplementada con carbohidratos o ácido oleico sobre el transcriptoma del tejido adiposo en las razas ibérica y duroc indican una respuesta más intensa en la primera, que se traduce esencialmente en cambios en los genes implicados en la inflamación, la respuesta inmune, el metabolismo de los lípidos y el engorde.

12. Existen efectos significativos de interacción raza*dieta sobre el transcriptoma del tejido adiposo de cerdos ibéricos y duroc, tanto de carácter cuantitativo como cualitativo, que podrían estar principalmente relacionados con el desarrollo de una respuesta inflamatoria en el cerdo ibérico que evoluciona de forma desacompasada o con diferente magnitud entre dietas.

CONCLUSIONS

1. Diet fatty acids, especially MUFA and PUFA, are deposited in tissues with little modifications, with tissues largely reflecting the composition of the diet received.

2. In Iberian pigs, in the short/medium and long terms, nutritional interventions with carbohydrates and FA induce transcriptional adaptations in the adipose and hepatic tissues.

3. Dietary carbohydrates stimulate the endogenous synthesis of saturated fat and SFA stimulate the synthesis of MUFA, thus suggesting an activating role of carbohydrate and SFA diets on *de novo* lipogenesis and desaturation of FA.

4. Effect of the growth period on the expression of genes related to lipid metabolism is large. Lipogenic gene expression increases with age. In contrast, the *PPARG* gene shows increased expression in growing animals in agreement with its role as regulator of adipogenesis, which occurs in early development stages.

5. In the Iberian pig adipose tissue, after an 18h-fasting period, transcriptional adaptation is only observed for the regulator PPARG. A longer fasting period (24h) is needed to induce an adaptation in the function of the main lipogenic genes.

6. During fasting, *de novo* lipogenesis in adipose tissue is more persistent in Iberian pigs than in Duroc pigs, which can be considered a characteristic trait associated with the fatty phenotype of these animals.

7. The regulation of lipolytic genes in adipose tissue, by factors such as breed, feeding status and diet supplementation, is very complex and subject to intricate interactions.

8. Effect of dietary supplementation with FA on lipid metabolism is moderate and variable depending on other factors such as breed or fasting, highlighting the complexity of its regulation and the difficulty of its study.

9. Breed has a profound effect on the adipose tissue transcriptome. Most transcriptome differences found between the Iberian and Duroc breeds are related to growth, extracellular matrix formation, lipid and carbohydrate

metabolism and remarkably to the inflammatory and immune response. Some of these differences may be related to the higher appetite of Iberian animals.

10. Adipose tissue of growing Iberian pigs is characterized by a transcriptional profile compatible with the development of low-grade inflammation and an insulin resistance syndrome, in accordance with their high fat deposition since early stages of development. These results confirm the interest of the Iberian pig as a biomedical model for studies on obesity, metabolic disorders and adipose tissue inflammation.

11. The effects of diets supplemented with carbohydrates or oleic acid on the adipose tissue transcriptome in the Iberian and Duroc breeds indicate a more intense response in the Iberian breed, which is essentially translated into changes in the genes involved in inflammation, immune response, lipid metabolism and fattening.

12. There are significant breed*diet interaction effects on adipose tissue transcriptome in Iberian and Duroc pigs, both quantitative and qualitative, which could be mainly related to the development of an inflammatory response in the Iberian pig that evolves in an uncoupled way or with different magnitude between diets.

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7. ANEXO 1: MATERIAL SUPLEMENTARIO

CAPITULO 2. Respuesta transcripcional de genes del metabolismo lipídico en tejido adiposo de cerdos ibéricos alimentados con una dieta enriquecida en ácido oleico versus carbohidratos

Table S1: Information on the primer pairs used for quantitative real-time PCR analysis, gene details and PCR efficiency (Eff).

CAPITULO 4. Efectos de la raza, la dieta y la interacción en el transcriptoma del tejido adiposo de cerdos Ibéricos y Duroc alimentados con diferentes fuentes de energía.

Table S1: Diet composition

Table S2: Primer design for qPCR validation

Table S3: Differentially expressed genes for the breed effect

Table S4: Canonical pathways enriched in the set of differentially expressed genes affected by breed

Table S5: Biological functions enriched in the set of differentially expressed genes affected by breed

Table S6: Regulators predicted for the set of differentially expressed genes affected by breed

Table S7: Differentially expressed genes for the diet effect in Iberian breed

Table S8: Differentially expressed genes for the diet effect in Duroc breed

Table S9: Canonical pathways enriched in the sets of differentially expressed genes affected by diet

Table S10: Biological functions enriched in the sets of differentially expressed genes affected by diet

Table S11: Regulators predicted for the sets of differentially expressed genes affected by diet

Table S12: DESeq2 results for the interaction breed*diet effect

8. ANEXO 2: ARTÍCULOS RELACIONADOS

8.1. Dietary energy source largely affects tissue fatty acid composition but has minor influence on gene transcription in Iberian pigs

Óvilo, C., Benítez, R., Fernandez, A., Isabel, B., Nuñez, Y., Fernandez, A.I., Rodríguez, C., Daza, A., Silió, L., López-Bote, C., 2014. *Journal of Animal Science* 92, 939-954

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Dietary energy source largely affects tissue fatty acid composition but has minor influence on gene transcription in Iberian pigs

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ABSTRACT: A trial was performed to compare the effects of different dietary sources of MUFA on the fatty acid (FA) composition, lipid metabolism, and gene transcription in different tissues of Iberian pigs. Twenty-seven Iberian male pigs of 28 kg live weight (LW) were divided in 2 groups and fed with 1 of 2 isocaloric diets: a standard diet with carbohydrates as energy source (CH) and a diet enriched with high-oleic sunflower oil (HO). Ham adipose tissue was sampled by biopsy at 44 and 70 kg LW. At 110 kg LW pigs were slaughtered and backfat, loin, and liver tissues were sampled. Animals of the HO group showed higher MUFA content and lower SFA in all the analyzed tissues ($P < 0.001$). These main effects were established early during the treatment and increased only slightly along time. Small diet effects were also detected on PUFA, which showed differences according to sampling time, tissue, and lipid fraction. Effects of diet on gene expression were explored with a combined approach analyzing adipose tissue transcriptome and quantifying the expression of a panel of key genes implicated in lipogenesis and lipid metabolism processes in backfat, muscle, and liver. Backfat transcriptome showed small effects of diet on gene expression, in number and magnitude. According to the posterior probabilities (*PP*) of the probe-specific expression differences between dietary groups ($PP < 0.01$), 37 genes were considered differentially expressed (DE). Gene ontology allowed relating them with several biological functions including lipid metabolic processes. Quantitative PCR confirmed several DE genes in adipose tissue (*RXRG*, *LEP*, and *ME1*; $P < 0.0001$, $P < 0.05$, and $P < 0.0001$, respectively), but no DE gene was found in loin or liver tissues. Joint results agree with a metabolic adjustment of adipose tissue FA levels by the subtle effect of the diet on the regulation of several lipid metabolism pathways, mainly FA oxidation and prostanoid synthesis, with *LEP*, *RXRG*, and *PTGS2* genes playing mayor roles.

Key words: fatty acid profile, gene expression, Iberian pig, nutrition, oleic acid

8.2. Prenatal programming in an obese swine model: sex-related effects of maternal energy restriction on morphology, metabolism and hypothalamic gene expression

Óvilo, C., González-Bulnes, A., Benítez, R., Ayuso, M., Barbero, A., Pérez-Solana, M.L., Barragán, C., Astiz, S., Fernández, A., López-Bote, C., 2014. *British Journal of Nutrition* 111, 735-746

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Prenatal programming in an obese swine model: sex-related effects of maternal energy restriction on morphology, metabolism and hypothalamic gene expression

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Abstract

Maternal energy restriction during pregnancy predisposes to metabolic alterations in the offspring. The present study was designed to evaluate phenotypic and metabolic consequences following maternal undernutrition in an obese pig model and to define the potential role of hypothalamic gene expression in programming effects. Iberian sows were fed a control or a 50 % restricted diet for the last two-thirds of gestation. Newborns were assessed for body and organ weights, hormonal and metabolic status, and hypothalamic expression of genes implicated in energy homeostasis, glucocorticoid function and methylation. Weight and adiposity were measured in adult littermates. Newborns of the restricted sows were lighter ($P < 0·01$), but brain growth was spared. The plasma concentration of TAG was lower in the restricted newborns than in the control newborns of both the sexes ($P < 0·01$), while the concentration of cortisol was higher in females born to the restricted sows ($P < 0·04$), reflecting a situation of metabolic stress by nutrient insufficiency. A lower hypothalamic expression of anorexigenic peptides (*LEPR* and *POMC*, $P < 0·01$ and $P < 0·04$, respectively) was observed in females born to the restricted sows, but no effect was observed in the males. The expression of *HSD11B1* gene was down-regulated in the restricted animals ($P < 0·05$), suggesting an adaptive mechanism for reducing the harmful effects of elevated concentrations of cortisol. At 4 and 7 months of age, the restricted females were heavier and fatter than the controls ($P<0·01$). Maternal feed restriction induces asymmetrical growth retardation and metabolic alterations in the offspring. Differences in gene expression at birth and higher growth and adiposity in adulthood suggest a female-specific programming effect for a positive energy balance, possibly due to overexposure to endogenous stress-induced glucocorticoids.

Key words: Prenatal programming: Energy balance: Fatness and obesity: Hypothalamic gene expression

The obesity epidemic is becoming one of the most important public health problems in many parts of the world, as it is associated with an increased risk of multiple chronic diseases, including several of the major causes of death and disability in the developed world (diabetes, CVD, stroke, hypertension and certain cancers). A recent study has estimated a 30 % increase in the prevalence of obesity and a 130 % increase in the prevalence of severe obesity over the next two decades[1].

The aetiology of obesity is complex as it is a multifactorial condition in which genetic, environmental and interaction factors are involved[2–4]. Among the environmental signals, nutritional status is known to have a profound impact on the development of obesity, not only during the development of the obese phenotype but also at earlier stages. In fact, it has been hypothesised that a deficient nutritional environment during the critical period of perinatal development programmes whole-body energy homeostasis for optimal survival under nutritionally deficient conditions[5–7]. When prenatal adaptations are mismatched with the environment that the individual confronts later in life, they may lead to metabolic alterations[8]. This process is known as 'prenatal/fetal programming' or 'developmental origins of health and disease'[9]. Epigenetic processes are known to be involved in the mechanisms responsible for the programming of body weight (BW) homeostasis[10,11], in agreement with the thrifty epigenotype theory, which states that DNA sequence polymorphisms may play

Abbreviations: BW, body weight; *LEPR*, leptin receptor; *POMC*, pro-opiomelanocortin.

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8.3. Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics

Óvilo, C., Benítez, R., Fernandez, A., Nuñez, Y., Ayuso, M., Fernandez, A., Rodríguez, C., Isabel, B., Rey, A., López-Bote, C., Silio, L., 2014. *BMC Genomics* 15, 413

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BMC Genomics

RESEARCH ARTICLE Open Access

Longissimus dorsi transcriptome analysis of purebred and crossbred Iberian pigs differing in muscle characteristics

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Abstract

Background: The two main genetic types in Iberian pig production show important phenotypic differences in growth, fattening and tissue composition since early developmental stages. The objective of this work was the evaluation of muscle transcriptome profile in piglets of both genetic types, in order to identify genes, pathways and regulatory factors responsible for their phenotypic differences. Contemporary families coming from pure Iberian pigs (IB) or from crossing with Duroc boars (DU×IB) were generated. Piglets (14 from each genetic type) were slaughtered at weaning (28 days) and *longissimus dorsi* was sampled for composition and gene expression studies. RNA was obtained and hybridized to Affymetrix *Porcine Genechip* expression arrays.

Results: Loin muscle chemical composition showed significant differences between genetic types in intramuscular fat content (6.1% vs. 4.3% in IB and DUxIB animals, respectively, $P = 0.009$) and in saturated ($P = 0.019$) and monounsaturated fatty acid proportions ($P = 0.044$). The statistical analysis of gene expression data allowed the identification of 256 differentially expressed (DE) genes between genetic types (FDR < 0.10), 102 upregulated in IB and 154 upregulated in DU×IB. Transcript differences were validated for a subset of DE genes by qPCR. We observed alteration in biological functions related to extracellular matrix function and organization, cellular adhesion, muscle growth, lipid metabolism and proteolysis. Candidate genes with known effects on muscle growth were found among the DE genes upregulated in DU×IB. Genes related to lipid metabolism and proteolysis were found among those upregulated in IB. Regulatory factors (RF) potentially involved in the expression differences were identified by calculating the *regulatory impact factors*. Twenty-nine RF were found, some of them with known relationship with tissue development (*MSTN*, *SIX4*, *IRX3*), adipogenesis (*CEBPD, PPARGC1B*), or extracellular matrix processes (*MAX*, *MXI1*). Correlation among the expression of these RF and DE genes show relevant differences between genetic types.

Conclusion: These results provide valuable information about genetic mechanisms determining the phenotypic differences on growth and meat quality between the genetic types studied, mainly related to the development and function of the extracellular matrix and also to some metabolic processes as proteolysis and lipid metabolism. Transcription factors and regulatory mechanisms are proposed for these altered biological functions.

Keywords: Iberian pig, Transcriptome, Genetic type, Transcription factors, Growth, Meat quality, Metabolism

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8.4. Haplotypic diversity of porcine LEP and LEPR genes involved in growth and fatness regulation

Pérez-Montarelo, D., Rodríguez, M.C., Fernández, A., Benítez, R.,Fabián García, F. Luis Silió, L., Fernández, A.I., 2015. *Journal of applied genetics*. DOI 10.1007/s13353-015-0284-7

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ANIMAL GENETICS • ORIGINAL PAPER

Haplotypic diversity of porcine *LEP* and *LEPR* genes involved in growth and fatness regulation

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Abstract The analysis of structural genetic variability in candidate genes can make it possible to analyse the selection footprint and deepen the understanding of the genetic basis of complex traits. The leptin (*LEP*) and its receptor (*LEPR*) porcine genes are involved in food intake and energy homeostasis, and polymorphisms associated to growth and fatness traits have been detected in both genes. The main objective of this study was to explore the genetic variability of the most polymorphic regions of both genes in a variety of pig populations and wild boars from diverse European and Asian origins. In total, 54 animals were included in the analyses, with a remarkable sampling of Spanish wild boars and Iberian pigs. The sequencing allowed the identification of 69 and 26 polymorphisms in *LEP* and *LEPR* genes, respectively. Neighbour-joining trees built for the 69 haplotypes identified in the *LEP* and the 24 haplotypes detected in the *LEPR* showed the known genetic divergence between European and Asian pig breeds. A high variability of the *LEP* was detected in the different analysed populations providing new data for the existence of two domestication centres in Asia. In comparison to the *LEP* gene, the *LEPR* showed a lower variability, especially in the Iberian breed that showed no variability. Moreover, results of the Hudson-Kreitman-Aguadé neutrality test support a possible selection event of the *LEPR* gene region in this breed, potentially related with its leptin resistance pattern and good adaptation to a traditional extensive production system with strong seasonal changes of feeding resources.

Keywords Haplotype · Iberian pig · *LEP* · *LEPR* · mtDNA · Selection

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Introduction

The genetic variability of livestock species has been shaped by diverse forces such as multiple domestication events, population admixture, natural selection and selective breeding. Since the beginning of the 1990s, molecular data have played an essential role in surveying the genetic variation within and between breeds of farm animals (Frankham et al. 2002). Most of the studies performed on pigs have been conducted using specific mitochondrial DNA (mtDNA) regions (Giuffra et al. 2000; Kim et al. 2002; Alves et al. 2003; Luetkemeier et al. 2010) or microsatellites markers. (Laval et al. 2000; Gama et al. 2013). Recently, a phylogenic analysis of complete genome sequences of wild boars and Asian and Western domestic pigs substantiates the hypothesis that pigs were independently domesticated in Eurasia and East Asia and supports the Asian influence in most of the cosmopolitan European and American breeds (Groenen et al. 2012). Moreover, the new massive parallel sequencing technologies have allowed for the identification of some candidate regions within the porcine genome that putatively have been under selection for diverse goals (Amaral et al. 2011; Wilkinson et al. 2013).

The analysis of the genetic variability in particular candidate genes related to quantitative traits can deepen the understanding of the genetic basis of such complex traits (D'Andrea et al. 2008; Yang et al. 2012). Because modern selective breeding towards leaner pigs must have dramatically affected

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8.5. Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs

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Long term vitamin A restriction improves meat quality parameters and modifies gene expression in Iberian pigs[1]

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ABSTRACT: Vitamin A is a key regulator of gene expression, influencing adipogenesis and lipid metabolism in animal tissues. This experiment was conducted to assess the effect of dietary vitamin A level and administration time on productive traits, intramuscular fat (IMF) content in ham muscles, tissue fatty acid composition, and expression of a panel of adipogenic and lipogenic candidate genes in Iberian pigs. Sixty piglets of 16.3 kg (SD = 2.5 kg) live weight (LW) were either fed a vitamin A–enriched diet (10,000 IU vitamin A/kg; CONTROL, $n = 20$) or a diet without supplemented vitamin A, applied from 16.3 kg (SD = 2.5 kg; early restriction group, ER, $n = 20$) or from an average weight of 35.8 kg (SD = 3.1 kg; late restriction group, LR, $n = 20$). Two slaughters were performed when pigs reached the averaged weights of 101.4 (SD = 4.1 kg) and 157.9 kg LW (SD = 7 kg) and samples from liver, heart, and backfat were obtained in both sacrifice times. In addition, ham subcutaneous fat and *Semimembranosus* (SM) and *Biceps Femoris* (BF) muscles were sampled at the last sacrifice. Dietary vitamin A level produced no effect on carcass traits in any of the harvests, while a small effect was observed on fatty acid composition in backfat at 101.4 kg LW. However, at 157.9 kg LW, the ER and LR groups showed higher MUFA content and lower SFA content in backfat, ham fat, and IMF ($P < 0.01$). In IMF, a decrease in n-6/n-3 PUFA ratio was observed in the restricted groups ($P < 0.005$). Intramuscular fat content in SM muscle was greater ($P < 0.05$) in the ER group than in the CONTROL and LR groups, while no difference was detected in BF muscle. Little effect of dietary vitamin A was observed in liver. Regarding changes in gene expression, *ACSL4*, *CEBPB*, and *IGF1* genes were upregulated ($P < 0.0001$, $P < 0.0001$, and $P < 0.05$, respectively) in the ER group in hepatic tissue, whereas *CRABPII* and *SCD* genes were upregulated ($P < 0.05$) in the same group in adipose tissue. On the other hand, *RXRG* was downregulated ($P < 0.05$) in the ER group in adipose tissue. Results found in this experiment show that long-term restriction of dietary vitamin A has a positive effect on nutritional and sensorial parameters of ham meat. Moreover, gene expression results were consistent with the vitamin A transcriptional regulation of adipogenesis and lipogenesis and with the changes observed in meat and fat composition.

Key words: fatty acid composition, gene expression, Iberian pig, meat quality, vitamin A

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INTRODUCTION

Intramuscular fat (**IMF**) content and composition are determinant factors for meat quality (Wood et al., 2008). Meat flavor, tenderness, and juiciness are mainly dependent on fat presence and compo-

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8.6. Comparative analysis of muscle transcriptome between pig genotypes identifies genes and regulatory mechanisms associated to growth, fatness and metabolism

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RESEARCH ARTICLE

Comparative Analysis of Muscle Transcriptome between Pig Genotypes Identifies Genes and Regulatory Mechanisms Associated to Growth, Fatness and Metabolism

Miriam Ayuso[1], Almudena Fernández[2], Yolanda Núñez[2], Rita Benítez[2], Beatriz Isabel[1], Carmen Barragán[2], Ana Isabel Fernández[2], Ana Isabel Rey[1], Juan F. Medrano[4], Ángela Cánovas[4¤], Antonio González-Bulnes[3], Clemente López-Bote[1], Cristina Ovilo[2]*

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Abstract

Iberian ham production includes both purebred (IB) and Duroc-crossbred (IBxDU) Iberian pigs, which show important differences in meat quality and production traits, such as muscle growth and fatness. This experiment was conducted to investigate gene expression differences, transcriptional regulation and genetic polymorphisms that could be associated with the observed phenotypic differences between IB and IBxDU pigs. Nine IB and 10 IBxDU pigs were slaughtered at birth. Morphometric measures and blood samples were obtained and samples from *Biceps femoris* muscle were employed for compositional and transcriptome analysis by RNA-Seq technology. Phenotypic differences were evident at this early age, including greater body size and weight in IBxDU and greater *Biceps femoris* intramuscular fat and plasma cholesterol content in IB newborns. We detected 149 differentially expressed genes between IB and IBxDU neonates ($p < 0.01$ and Fold-Change > 1. 5). Several were related to adipose and muscle tissues development (*DLK1*, *FGF21* or *UBC*). The functional interpretation of the transcriptomic differences revealed enrichment of functions and pathways related to lipid metabolism in IB and to cellular and muscle growth in IBxDU pigs. Protein catabolism, cholesterol biosynthesis and immune system were functions enriched in both genotypes. We identified transcription factors potentially affecting the observed gene expression differences. Some of them have known functions on adipogenesis (*CEBPA*, *EGRs*), lipid metabolism (*PPARGC1B*) and myogenesis (*FOXOs*, *MEF2D*, *MYOD1*), which suggest a key role in the meat quality differences existing between IB and IBxDU hams. We also identified several polymorphisms showing differential segregation between IB and IBxDU pigs. Among them, non-synonymous variants were detected in

8.7. Using RNA-Seq SNP data to reveal potential causal mutations related to pig production traits and RNA editing

Martínez-Montes, A.M., Fernández, A., Pérez-Montarelo, D., Alves, E., Benítez, R., Núñez, Y., Óvilo, C., N. Ibáñez-Escriche, N., Folch, J.M., Fernández, A. I., 2016.

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Using RNA-Seq SNP data to reveal potential causal mutations related to pig production traits and RNA editing

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Summary

RNA-Seq technology is widely used in quantitative gene expression studies and identification of non-annotated transcripts. However this technology also can be used for polymorphism detection and RNA editing in transcribed regions in an efficient and cost-effective way. This study used SNP data from an RNA-Seq assay to identify genes and mutations underlying production trait variations in an experimental pig population. The hypothalamic and hepatic transcriptomes of nine extreme animals for growth and fatness from an (Iberian × Landrace) × Landrace backcross were analyzed by RNA-Seq methodology, and SNP calling was conducted. More than 125 000 single nucleotide variants (SNVs) were identified in each tissue, and 78% were considered to be potential SNPs, those SNVs segregating in the context of this study. Potential informative SNPs were detected by considering those showing a homozygous or heterozygous genotype in one extreme group and the alternative genotype in the other group. In this way, 4396 and 1862 informative SNPs were detected in hypothalamus and liver respectively. Out of the 32 SNPs selected for validation, 25 (80%) were confirmed as actual SNPs. Association analyses for growth, fatness and premium cut yields with 19 selected SNPs were carried out, and four potential causal genes (*RETSAT*, *COPA*, *RNMT* and *PALMD*) were identified. Interestingly, new RNA editing modifications were detected and validated for the *NR3C1*:g.102797 (ss1985401074) and *ACSM2B*:g.13374 (ss1985401075) positions and for the *COG3*:g3.4525 (ss1985401087) modification previously identified across vertebrates, which could lead to phenotypic variation and should be further investigated.

Keywords association, fatness, Iberian pig, premium cut yield, transcriptom

Introduction

The recent availability of massive sequencing technologies provides new tools for the search of causal genes and mutations. Several approaches, such as whole genome sequencing, exome capture and sequencing, chromatin immunoprecipitation sequencing and transcriptome sequencing, have been developed to answer different biological questions (Bai *et al.* 2012). In particular, RNA sequencing (RNA-Seq) technology is largely used in quantitative gene expression studies as a source of biological information to support the identification of causal mutations underlying the variation of complex traits (Hudson *et al.* 2012). RNA-seq methodology allows for a comprehensive analysis and quantification of all RNA types expressed in tissues or cells, including mRNA, non-coding RNA and small RNA (Wang *et al.* 2009). In comparison with gene expression microarrays, RNA-seq technology is able to detect transcripts expressed at low levels and alternative isoforms (Ferraz *et al.* 2008; Trapnell *et al.* 2009). During the last few years, the RNA-seq method has also been employed with farm animals and has helped in the selection of candidate genes related to important traits through the comparison of global gene expression profiles between groups of animals that differ in specific traits (i.e. Ramayo-Caldas *et al.* 2012; Pérez-Montarelo *et al.* 2014; Wang *et al.* 2015; Zhang *et al.* 2015).

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8.8. Developmental Stage, Muscle and Genetic Type Modify Muscle Transcriptome in Pigs: Effects on Gene Expression and Regulatory Factors Involved in Growth and Metabolism

Ayuso, M., Fernández, A., Núñez, Y., Benítez, R., Isabel, B., Fernández, A.I., Rey, A.I., González-Bulnes, A., Medrano, J.F., Cánovas, Á., López-Bote, C.J., Óvilo, C., 2016. *PLoS One* 11(12), e0167858

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RESEARCH ARTICLE

Developmental Stage, Muscle and Genetic Type Modify Muscle Transcriptome in Pigs: Effects on Gene Expression and Regulatory Factors Involved in Growth and Metabolism

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Abstract

Iberian pig production includes purebred (IB) and Duroc-crossbred (IBxDU) pigs, which show important differences in growth, fattening and tissue composition. This experiment was conducted to investigate the effects of genetic type and muscle (*Longissimus dorsi* (LD) *vs Biceps femoris* (BF)) on gene expression and transcriptional regulation at two developmental stages. Nine IB and 10 IBxDU piglets were slaughtered at birth, and seven IB and 10 IBxDU at four months of age (growing period). Carcass traits and LD intramuscular fat (IMF) content were measured. Muscle transcriptome was analyzed on LD samples with RNA-Seq technology. Carcasses were smaller in IB than in IBxDU neonates ($p < 0.001$), while growing IB pigs showed greater IMF content ($p < 0.05$). Gene expression was affected ($p < 0.01$ and Fold change > 1.5) by the developmental stage (5,812 genes), muscle type (135 genes), and genetic type (261 genes at birth and 113 at growth). Newborns transcriptome reflected a highly proliferative developmental stage, while older pigs showed upregulation of catabolic and muscle functioning processes. Regarding the genetic type effect, IBxDU newborns showed enrichment of gene pathways involved in muscle growth, in agreement with the higher prenatal growth observed in these pigs. However, IB growing pigs showed enrichment of pathways involved in protein deposition and cellular growth, supporting the compensatory gain experienced by IB pigs during this period. Moreover, newborn and growing IB pigs showed more active glucose and lipid metabolism than IBxDU pigs. Moreover, LD muscle seems to have more active muscular and cell growth, while BF points towards lipid metabolism and fat deposition. Several regulators controlling transcriptome changes in both genotypes were identified across muscles and ages (*SIM1*, *PVALB*, *MEFs*, *TCF7L2* or *FOXO1*), being strong candidate genes to drive expression and thus, phenotypic

8.9. Nutrigenomics in Farm Animals

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Journal of Investigative Genomics

Nutrigenomics in Farm Animals

Abstract

Nutrigenomics and nutrigenetics are new disciplines that study the effects of food at the genetic level. Nutrigenomics studies how bioactive chemicals in foods and supplements affects animal metabolism by altering gene expression and unites many fields: nutrition, bioinformatics, molecular biology, genomics, functional genomics, epidemiology, and epigenomics. The use of multi-disciplinary tools provides new opportunities to investigate the complex interactions of the genome and the diet. These new approaches highlight the relevant role of genetics-nutrition interactions on different physiological and metabolic processes with a high impact on economically relevant traits as meat and milk quality, and the interest of multidisciplinary studies to face these new complex issues. The objective of this mini-review is to discuss the basic concepts, technical terms and technologies involved in Nutrigenomics studies and also to discuss the results of some published works that show how nutrition affects the expression of genes involved in lipid metabolism, oxidative processes and milk production and how nutritional intervention might change meat quality and tissue composition.

Keywords: Nutrigenomics; Transcriptomics; Gene expression; Nutrition and Farm animals

Mini Review

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Abbreviations: PUFA: Polyunsaturated Fatty Acid; PCR: Polymerase Chain Reaction; DNA: Deoxyribonucleic Acid; RNA: Ribonucleic Acid; SFA: Saturated Fatty Acid; MUFA: Monounsaturated Fatty Acid; SCD: Δ9-desaturase; FASN: Fatty Acids Synthase; STAT5: Signal Transducer and Activator of Transcription 5; CEBPA: CCAAT/Enhancer-Binding protein (alpha); CLA: Conjugated Linoleic Acid; ACOX1 : Acyl-coa Oxidase 1; ACSL4: Acyl-CoA Synthetase Long-chain family member 4; RXRG: Retinoid X Receptor Gamma; CEBPB: CCAAT/Enhancer Binding Protein (beta); IGF1: Insulin-Like Growth Factor 1; INSR: Insulin Receptor; CRABII: Cellular Retinoic Acid Binding Protein 2; IMF Intramuscular Fat; VE: Vitamin E; GP: Grape Pomace; CAT: Catalase; SCP2: Sterol Carrier Protein 2; HADH: 3-hydroxyacyl CoA Dehydrogenase; HMOX2: Heme-Oxygenase 2; SOD1: Superoxide Dismutase 1

Introduction

In recent years there has been an enormous development of molecular genetics techniques allowing the study of genome function on a large scale. These advances have been accompanied by a decrease in costs and greater accessibility, which has contributed to the development of new disciplines that would fall within the generic term "Nutritional Genomics". This nascent area studies the interactions of food and its components with the genome at the molecular, cellular and systemic levels. Nutritional Genomics is currently divided into two different fields of research:

"Nutrigenomics" has emerged as a novel and multidisciplinary research field in nutritional science that aims to elucidate how dietary nutrients can interact with genes affecting transcription factors, RNA and protein expression, cellular homeostasis and metabolite production (genome, transcriptome, proteome, metabolome). In this field, many studies have been carried out in human and mouse, with the main objective to study the molecular basis of diseases such as obesity, cardiovascular diseases and cancer, understanding them as a result of gene/diet type interactions [1,2]. In domestic animals these nutrigenomic studies are much less abundant and in general are focused on the study of a reduced number of genes especially related to a particular treatment, mainly referred to the energy content of the diet, or the content of some of its components: polyunsaturated fatty acids (PUFA) [3] protein [4] or L-carnitine [5].

"Nutrigenetics" studies how structural genetic variation affects the diet effects on phenotype. There are possibly thousands of gene polymorphisms which may result in minor deviations in nutritional biochemistry influencing the diet effects on metabolism. The ultimate objective of this applied discipline is to provide nutritional recommendations taking into account the genetic makeup of each individual, what in human nutrition is called "Personalized Nutrition". In domestic animals this discipline is practically unexplored.

Nutrigenomics plays an important role in integrating genomic approaches into nutrition research. Well over a decade ago, nutrigenomics diverged from mainstream nutritional science as a specific methodological and conceptual approach [6]. Over the course of this decade, the identity of nutrigenomics as an independent field of research was firmly established sometimes in opposition to "nutrition science". So far, in animal production, nutrition and genetics have mainly been used as independent disciplines without taking into account the genome-nutrition interactions on the various physiological and metabolic processes with transcendence on phenotype and production. The interest of the nutrigenomic studies is that they allow us to deepen in the

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Effects of lysine deficiency on carcass composition and activity and gene expression of lipogenic enzymes in muscles and backfat adipose tissue of fatty and lean piglets

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*The purpose of this study was to investigate potential mechanisms involved in fat deposition promoted by dietary lysine deficiency, particularly intramuscular fat (**IMF**), and differential responses between fatty and lean pigs. Carcass traits and lipogenic enzyme activities and gene expression levels in muscles and adipose tissue were investigated in Iberian (fatty) and Landrace × Large White (**LDW**) pigs under identical feeding level (based on body weight (**BW**)) and management conditions. Twenty-eight barrows of 10 kg initial BW, 14 per breed, were fed two isoproteic (200 g CP /kg DM) and isocaloric (14.7 MJ metabolizable energy/kg DM) diets with identical composition except for the lysine content (1.09% for diet adequate in lysine and 0.52% for diet deficient in lysine). At a BW of 25 kg, pigs were slaughtered. Compared with pigs fed the lysine-adequate diet, in both genotypes lysine-deficient diet led to lower carcass protein concentration, lower relative proportions of leaner components (loin, ham and shoulder; $P < 0.01$), and higher carcass fatty components and carcass lipid concentration ($P < 0.001$). Irrespective of diet, the activity and gene expression of lipogenic enzymes (fatty acid synthase (**FAS**), malic enzyme (**ME**) and glucose-6-phosphate dehydrogenase (**G6PDH**)) were greater in Iberian than in LDW pigs, particularly in adipose tissue where transcriptional regulators involved in the control of adipogenesis and lipogenesis were also upregulated in Iberian animals. In backfat tissue, there was a small decrease induced by or no effects of lysine-deficient diet on the activity and gene expression of lipogenic enzymes, nor in gene expression levels of upstream regulators of lipogenesis and adipogenesis. In* longissimus *muscle, the activity of FAS, G6PDH and ME increased with lysine deficiency in both genotypes ($P < 0.01$) and an upregulation of gene expression of lipogenic enzymes was specifically observed in Iberian pigs ($P < 0.05$ to $P < 0.001$). In* biceps femoris *muscle of lysine-deficient pigs, the activity of FAS and ME enzymes increased,* ME1 *gene was upregulated (added to* FASN *gene in the case of Iberian pigs; $P < 0.01$ to $P < 0.001$) and* PPARA *gene was downregulated ($P < 0.05$). The results show that in both fatty and lean pigs, the effect of lysine deficiency on lipid metabolism was tissue-specific, with an activation of lipogenesis in* longissimus *and* biceps femoris *muscle but no apparent stimulation in backfat adipose tissue. Suitable feeding protocols including lysine-deficient diets should be designed for each pig type in order to increase intramuscular lipids without penalizing the growth of lean carcass components.*

Keywords: dietary lysine, fat depots, lipogenesis, pig genotype, RNA

Implications

Specific nutritional strategies involving dietary lysine restriction can be developed for fattening fatty and lean pigs to increase **IMF** with expected positive consequences on meat quality. The biochemical mechanisms that contribute to the increase of fat in these conditions seem to differ among tissues such as subcutaneous backfat and muscle. Further studies including time–course measurements can contribute to develop suitable strategies for the modulation of fat content in pork meat.

Introduction

A major factor affecting meat organoleptic characteristics is the amount and composition of intramuscular fat (IMF) (Wood *et al.*, 2008), and a positive relationship between the acceptability or the tenderness of pork and IMF content

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8.11. Analysis of porcine IGF2 gene expression in adipose tissue and its effect on fatty acid composition

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RESEARCH ARTICLE

Analysis of porcine *IGF2* gene expression in adipose tissue and its effect on fatty acid composition

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Abstract

IGF2:g.3072G>A polymorphism has been described as the causal mutation of a maternally imprinted QTL for muscle growth and fat deposition in pigs. The objective of the current work was to study the association between the *IGF2:g.3072G>A* polymorphism and the *IGF2* gene expression and its effect on fatty acid composition in adipose tissue in different pig genetic backgrounds. A *cis*-eQTL region associated with the *IGF2* mRNA expression in adipose tissue was identified in an eGWAS with 355 animals. The *IGF2* gene was located in this genomic interval and *IGF2g.3072G>A* was the most significant SNP, explaining a 25% of the gene expression variance. Significant associations between *IGF2:g.3072G>A* polymorphism and oleic (C18:1(n-9); p-value = $4.18x10^{-07}$), hexadecanoic (C16:1(n-9); p-value = $4.04x10^{-07}$), linoleic (C18:2(n-6); p-value = $6.44x10^{-09}$), α-linoleic (C18:3(n-3); p-value = $3.30x10^{-06}$), arachidonic (C20:4(n-6); p-value = $9.82x10^{-08}$) FAs and the MUFA/PUFA ratio (p-value = $2.51x10^{-9}$) measured in backfat were identified. Animals carrying the *A* allele showed an increase in *IGF2* gene expression and higher PUFA and lower MUFA content. However, in additional studies was observed that there could be other proximal genetic variants affecting FA composition in adipose tissue. Finally, no differences in the *IGF2* gene expression in adipose tissue were found between heterozygous animals classified according to the *IGF2:g.3072G>A* allele inherited from the father (A^PG^M or A^MG^P). However, pyrosequencing analysis revealed that there is imprinting of the *IGF2* gene in muscle and adipose tissues, with stronger differences among the paternally and maternally inherited alleles in muscle. Our results suggested that *IGF2:g.3072G>A* polymorphism plays an important role in the regulation of *IGF2* gene expression and can be involved in the fatty acid composition in adipose tissue. In both cases, further studies are still needed to deepen the mechanism of regulation of *IGF2* gene expression in adipose tissue and the *IGF2* role in FA composition.